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CHARACTERIZATION OF *RADOPHOLUS SIMILIS* RESISTANCE IN *MUSA* SPP. WITH EMPHASIS ON PHYTOCHEMICAL ANALYSIS

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Cover illustration: Top: Light microscopic image of *Musa* genotypes Long Tavoy (left) and Yangambi km5 (right) roots showing the accumulation of phenolic phytoalexins due to *R. similis* infection.
Bottom: Light microscopic image of a moulting *Radopholus similis* juvenile that had killed by the uptake of phenylphenalenone type-pheytoalexin anigorufone during a laboratory bio-assay.

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^{13}C	carbon 13
^{1}H NMR	proton nuclear magnetic resonance
Acetone- d_6	deuterated acetone
aHPLC	analytical HPLC
ANOVA	analysis of variance
AU	absorbance units
C	carbon
$CHCl_3$	chloroform
CO_2	carbon dioxide
CRD	completely randomized design
cv.	cultivar
DAD	diode array detector
DAI	days after inoculation
DBCP	1,2-dibromo-3-chloropropane
DD	dichloropropane-dichloropropene
DNA	deoxyrinonucleic acid
DPBA	diphenylboric acid 2-aminoethyl ester
EDB	ethylene dibromide
EPPO	European and Mediterranean Plant Protection Organization
FITC	fluorescein isothiocyanate
G. Naine	Grande Naine
GN	Grande Naine
H_2O	water
ha	hectare
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HR	hyper sensitive response
HSD	honestly significant difference
IAA	indole acetic acid
IC_{50}	inhibitive concentration affecting 50% of nematode population
ITC	International Transit Centre, K.U.Leuven
ITO	indium tin oxide
J2	second-stage juveniles
$KMnO_4$	potassium permanganate
LC-MS	liquid chromatography coupled with mass spectrometry
LDI-MSI	laser desorption/ionisation mass spectrometry imaging
LTGA	lignothioglycolic acid
m/z	mass-to-charge ratio
MALDI	matrix assisted laser desorption/ionisation mass spectrometry imaging
MS	mass spectrometry

n	number of repetitions
Na_2CO_3	sodium carbonate
NaCl	sodium chloride
NaOCl	sodium hypochlorite
NaOH	sodium hydroxide
Nd:YAG	neodymium-doped yttrium aluminium garnet
NH_3	ammonia
NMR	nuclear magnetic resonance spectroscopy
PAL	phenylalanine ammonia lyase
Pf	final nematode population density
pHPLC	preparative HPLC
Pi	initial nematode population density
ppm	parts per million
PR	pathogenesis related
PTFE	polytetrafluoroethylene
RBD	randomized block design
Rf	reproduction factor
R_f	retention factor
Ri T-DNA	root-inducing transferred-DNA
RNA	ribonucleic acid
Rr	reproduction ratio
R_t	retention time
SAR	systemically acquired resistance
SDS	sodium dodecyl sulphate
TAE	tannic acid equivalents
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TMS	tetramethylsilane
TRIS	tris(hydroxymethyl)aminomethane
UPLC-MS	ultra performance liquid chromatography-mass spectrometry
UV	ultraviolet
UV-Vis	ultraviolet-visible spectroscopy
v/v	volume per volume
w/v	weight per volume
WAI	weeks after infection
WAT	weeks after transplantation
YKm5	Yangambi Km5

SUMMARY

The burrowing nematode, *Radopholus similis* (Cobb, 1893) Thorne, 1949 is considered as the most damaging nematode species in commercial banana plantations. Nematicides have been intensively used to control plant-parasitic nematodes. However, many effective nematicides have been withdrawn from the market due to their adverse effects on the environment, non-target organisms and the accumulation of toxic residues in the food chain.

The use of resistant cultivars is an efficient and economical alternative approach for controlling nematode populations. Unraveling the mode(s) of action or gaining in-depth knowledge on the mechanism(s) of host resistance to a nematode may provide information that can be used either to improve the efficacy of the use of the resistance or to assist in the faster selection or breeding of resistant cultivars. The main objective of our study was to characterise the mechanism(s) of resistance to *R. similis* in *Musa* spp.

To achieve this objective, seven newly reported *R. similis*-resistant *Musa* genotypes were selected and their response to *R. similis* infection was verified under greenhouse conditions. The host responses were compared with the well-known *R. similis*-resistant reference cultivars Yangambi km5, Pisang Jari Buaya and the susceptible cultivar Grande Naine. The host response of these *Musa* genotypes to the root-knot nematode *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 infection was also evaluated to examine if the *R. similis*-resistant genotypes are also resistant to *M. incognita*.

Four *Musa* genotypes Long Tavoy, Saba, Pisang Mas and Pora Pora expressed resistance to *R. similis*. Marau expressed a partial resistance to *R. similis*. The host response of Kokopo was inconclusive and Gia Hui was susceptible to *R. similis*. We consider the use of tissue culture-derived plants and highly pathogenic population of *R. similis* as the reasons for susceptibility of the previously reported *R. similis*-resistant genotype Gia Hui. For the first time, Vudu Papau and Pisang Mas were identified as resistant to *M. incognita* based on the final nematode population density. Pisang Mas is identified as a *Musa* genotype with combined resistance to both *R. similis* and *M. incognita*. This is the first time that a *Musa* genotype has been found as resistant to these two major banana root pathogens. Three *R. similis* resistant *Musa* genotypes Long Tavoy, Saba and Yangambi km5 were shortlisted for characterization of *R. similis* resistance in comparison with the susceptible reference cultivar Grande Naine.

Our first major specific objective was to find out at which phase of the nematode-plant interactions (*i.e.* pre- or post-infection) the resistance to *R. similis* was active in the *Musa* genotypes. To achieve this specific objective, an autotrophic *in vitro* model system was developed to compare *R. similis* attraction, migration towards and penetration of the *R. similis*-resistant and susceptible *Musa* genotypes. A novel two-compartment system was developed to examine the attraction of *R. similis* to either Grande Naine or Yangambi km5 when both *Musa* genotypes were present.

Plant root growth in the autotrophic *in vitro* system was good with well-developed secondary and tertiary roots. The autotrophic *in vitro* system was an advantageous model system to study nematode attraction towards and penetration in the roots of banana. This include good plant and root growth in the autotrophic system and ability to observe the living plant roots under microscope without compromising the easy handling, short duration and aseptic conditions of strict *in vitro* model systems. No significant differences were observed in the percentage of *R. similis* migrated towards the roots of the Grande Naine (susceptible) and Yangambi km5 (resistant) at 3, 4 and 6 h after inoculation. The percentages of females that had penetrated the roots of Yangambi km5 and Grande Naine were not significantly different from each other at 1 and 2 days after inoculation. The results of the two-compartment system showed no significant differences in the percentage of *R. similis* females migrated towards the roots of resistant and susceptible genotypes when they could access both roots at the same time. Hence, *R. similis* females showed no preference to migrate towards the roots of either the resistant or susceptible *Musa* genotype when a choice was given.

Two greenhouse experiments were conducted on the penetration, development and reproduction of *R. similis* on three resistant (Long Tavoy, Saba and Yangambi km5) and one susceptible (Grande Naine) *Musa* genotypes. *Radopholus similis* penetration rate was checked at 4, 8 and 12 days after inoculation. The nematode development and reproduction were checked at 26 days after inoculation.

No significant differences were observed between the numbers of nematodes that had penetrated the four *Musa* genotypes at 4 and 8 days after inoculation. Eggs were observed in Grande Naine but not in any of the three resistant *Musa* genotypes at 8 days after inoculation. Significantly lower number of eggs was observed in the roots of a resistant genotype Long Tavoy at 12 days compared to Grande Naine. The number of eggs, males, females, number of adults and juveniles and final population density were significantly higher in the susceptible genotype Grande Naine compared to the three *R.*

similis-resistant *Musa* genotypes at 26 days after inoculation. The post-infectional nematode development and reproduction were severely impaired in the resistant *Musa* genotypes. Hence, it appears that the mechanism of resistance in the investigated *Musa* genotypes to *R. similis* is induced after nematode penetration and that preformed host resistance factors do not function strongly against the nematode attraction and migration towards the roots, and penetration of the roots.

The second major specific objective of our study was to identify the phytochemicals involved in the resistance to *R. similis* in the resistant *Musa* genotypes. A preliminary phytochemical profiling was performed to assess the involvement of lignin and phenols in the resistance of the three *R. similis*-resistant *Musa* genotypes in comparison with Grande Naine.

The uninfected plants of all *Musa* genotypes sampled at 6 weeks after inoculation showed intense lignifications in their vascular bundle compared to the plants sampled at 3 weeks after infection. This showed that the older roots are more extensively lignified in the secondary cell walls than the younger roots. Nematode infection has significantly increased the lignin content of Yangambi km5 roots at 6 weeks after inoculation. In all four *Musa* genotypes, lignification started at the endodermis, extended to the peripheral tissues of vascular system especially the xylem walls, xylem-accompanying parenchyma cells and eventually progress to the vascular parenchyma (sclerenchyma) cells in the centre. The cortex and aerenchyma cells were lignified to a very small extent. Extensive lignification is not associated with the cortex cells that are directly involved in the defense to *R. similis*. This shows that the increased lignification is only a general defense response to protect the vascular bundle to reduce damage to the plants. Hence it appears that the lignification is more associated with the plant's tolerance to *R. similis* preventing damage to the plants than resistance to nematode development, reproduction and multiplication. Histochemical localisation of total phenols by staining with toluidine blue showed no preformed phenolic cells in the cortex of the *R. similis*-resistant and susceptible *Musa* genotypes. Phenolic substances were major constituents of the nematode infected necrotic cells. The Folin-Ciocalteu assay showed that the nematode infection has almost doubled the total phenols contents in all *Musa* genotypes at 3 weeks after infection and in the resistant genotypes at 6 weeks after inoculation. The enhanced synthesis of phenols could be due to the biosynthesis or accumulation of secondary metabolites such as phytoalexins in the nematode infection sites.

In the final part of characterizing the nematode resistance in *Musa* genotypes, a combination of analytical techniques namely high performance liquid chromatography (HPLC), Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and ultra performance liquid chromatography coupled with mass spectrometry (UPLC-MS) was used to identify the secondary metabolites that are induced in nematode infection sites. The secondary metabolites were localised to study their distribution using matrix-free laser desorption/ionisation mass spectrometric imaging (LDI-MSI) techniques.

The HPLC and ^{1}H NMR analysis structurally identified nine phenylphenalenone-type phytoalexins and the UPLC-MS analysis identified one additional phenylphenalenone-type phytoalexin from *R. similis*-infected necrotic tissues of the banana roots. Our results provided clear evidence for the induction of phenylphenalenone-type secondary metabolites in *Musa* spp. in response to *R. similis* infection. These compounds were absent in the uninfected control roots. The phenylphenalenones showed a highly localised presence only in the nematode-infected necrotic regions of the banana roots. Observation of the lesions also showed that, in Yangambi km5, the lesions are small, discontinuous and non-expanding as in hypersensitive lesions. But, in Grande Naine, the lesions were large, tunnel-like and covered larger areas of the infected roots. Anigorufone was identified as the most abundant phenylphenalenone-type secondary metabolite present in the necrotic lesions of *R. similis*-resistant and susceptible cultivars. But, the concentration of anigorufone per unit area was very high in Yangambi km5 compared to Grande Naine. The higher concentration of anigorufone and other phenylphenalenone-type phytoalexins localised in few cells in the *R. similis*-resistant cultivar can create a more toxic cellular environment to the nematode compared to Grande Naine.

The anti-nematode properties of the identified phenylphenalenones were assessed by *in vitro* bio-assays on nematode motility inhibition and nematode mortality. Eleven out of thirteen tested phenylphenalenones were inhibitive to *R. similis* motility. Our results are the first evidence for the anti-nematode properties of phenylphenalenones.

Anigorufone, 4-hydroxy-2-methoxy-9-phenylphenalenone and isoanigorufone were remarkably powerful anti-nematode compounds tested in our study in that they caused quiescence to more than 75% of *R. similis* juveniles and adults. Hydroxyanigorufone and monohydroxyanigorootin were also highly inhibitive to *R. similis* motility starting from 24 h of incubation and the effect was either consistent or increased over time. Anigorufone was the most powerful anti-nematode compound assayed. The motility inhibition

concentration of anigorufone required to cause quiescence to 50% of the tested *R. similis* (IC_{50}) was only 23 ppm at 72 h of incubation. At higher concentrations of anigorufone such as 50, 100 and 150 ppm, nematodes ingested large amounts of the compound causing mortality of the nematodes. The ingested anigorufone was found accumulated in the nematode guts of all life stages of nematodes. Elaborated future bio-assays using different combinations of the identified compounds will enhance the understanding on the synergism or antagonism of the compounds.

Future studies could explore the possibilities of enhancing the cellular concentration and improved localisation of anigorufone and other phenylphenalenones in the roots of *R. similis*-susceptible commercially successful banana cultivars such as Grande Naine.

SAMENVATTING

De tunnels gravende nematode *Radopholus similis* (Cobb, 1893) Thorne, 1949 wordt beschouwd als de meest schadelijke soort in commerciële bananenplantages. Nematiciden werden intensief gebruikt om plantenparasitaire nematoden te bestrijden. Vele effectieve nematiciden zijn echter uit de markt gehaald wegens hun kwalijke effecten op het milieu en niet-doelorganismen en de accumulatie van toxische residuen in de voedselketen.

Het gebruik van resistente cultivars vormt een efficiënte en voordelige alternatieve benadering voor de controle van nematodenpopulaties. Het ontrafelen van de werkingsmechanismen en een dieper inzicht in de mechanismen van gastheerresistentie tegenover nematoden kan informatie aanreiken die gebruikt kan worden om de efficiëntie van resistentie te verbeteren, of bijdragen aan de snellere selectie en kweek van resistente cultivars. Het hoofddoel van onze studie was dan ook om de werkingsmechanismen van de resistentie tegenover *R. similis* in *Musa* spp. te karakteriseren.

Om dit doel te bereiken, werden zeven *Musa* genotypes geselecteerd waarvan de resistentie tegen *R. similis* recent gerapporteerd werd, en hun respons tegenover *R. similis* infectie werd geverifieerd onder serrecondities. De gastheerrespons werd vergeleken met die van de welgekende *R. similis*-resistente referentiecultivars Yangambi km5, Pisang Jari Buaya en met het gevoelige cultivar Grande Naine. De gastheerrespons van deze *Musa* genotypes tegen infectie door de wortelknobbelnematode *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 werd eveneens geëvalueerd, om te onderzoeken of de *R. similis*-resistente genotypes ook resistent zijn tegen *M. incognita*.

Vier *Musa* genotypes Long Tavoy, Saba, Pisang Mas and Pora Pora vertoonden resistentie tegen *R. similis*. Marau vertoonde een gedeeltelijke resistentie tegen *R. similis*. De gastheerrespons van Kokopo was onduidelijk en Gia Hui was gevoelig voor *R. similis*. Het gebruik van planten afkomstig van weefselcultuur en de hoge pathogeniciteit van de *R. similis* populatie kunnen aangehaald worden als redenen voor de gevoeligheid van het genotype Gia Hui, dat eerder gerapporteerd werd als zijnde *R. similis*-resistent. Vudu Papau en Pisang Mas werden voor het eerst geïdentificeerd als resistent tegen *M. incognita*, gebaseerd op de finale densiteit van de nematodenpopulatie. Pisang Mas werd geïdentificeerd als *Musa* genotype met gecombineerde resistentie

tegen zowel *R. similis* als *M. incognita*. Het is de eerste keer dat een *Musa* genotype gevonden werd dat resistent bleek tegen deze twee belangrijke wortelpathogenen van banaan. Drie *R. similis* resistente *Musa* genotypes Long Tavoy, Saba en Yangambi km5 werden weerhouden voor karakterisatie van de *R. similis* resistentie in vergelijking met het gevoelige referentiecultivar Grande Naine.

Het eerste specifieke hoofddoel was om te ontdekken op welke fase van de nematode-planteninteractie (*i.e.* pre- of post-infectioneel) de resistentie tegen *R. similis* geactiveerd wordt in de *Musa* genotypes. Om dit specifieke hoofddoel te bereiken, werd een autotroof *in vitro* model ontwikkeld om *R. similis* attractie, migratie en penetratie in de *R. similis* resistente en gevoelige *Musa* genotypes te vergelijken. Een nieuw bicompartimenteel systeem werd ontwikkeld om de attractie van *R. similis* naar Grande Naine of Yangambi km5 te vergelijken wanneer beide Musa genotypes tegelijk aanwezig waren.

De planten in het autotrofe *in vitro* systeem vertoonden een goede wortelgroei, met goed ontwikkelde secundaire en tertiaire wortels. Het autotrofe *in vitro* systeem was een goed modelsysteem om de nematodenattractie en wortelpenetratie in banaan te bestuderen met diverse voordelen. Deze omvatten een goede plantengroei en wortelgroei in het autrotrofe systeem, de mogelijkheid om levende plantenwortels te observeren onder de microscoop, en de korte duur en aseptische condities van een strikt *in vitro* systeem. Er werden geen significante verschillen geobserveerd in het percentage *R. similis* nematoden die migreerden naar de wortels van Grande Naine (gevoelig) en Yangambi km5 (resistent) op 3, 4 and 6h na inoculatie. Het percentage vrouwelijke nematoden dat de wortels van Yangambi km5 en Grande Naine binnendrong was niet significant verschillend op 1 en 2 dagen na inoculatie. De resultaten van het bicompartimenteel systeem vertoonden geen significante verschillen in het percentage vrouwelijke *R. similis* dat migreerde naar de wortels van resistente of gevoelige genotypes, wanneer beide tegelijk konden bereikt worden. Vrouwelijke *R. similis* vertoonden dus geen voorkeur voor migratie in de richting van de wortels van resistente of gevoelige *Musa* genotypes wanneer zij de keuze kregen.

Twee serre-experimenten werden uitgevoerd om de penetratie, ontwikkeling en reproductie van *R. similis* te bestuderen op drie resistente (Long Tavoy, Saba en Yangambi km5) en één gevoelig (Grande Naine) *Musa* genotype. De penetratiegraad van *R. similis* werd bepaald op 4, 8 en 12 dagen na inoculatie. De nematodenontwikkeling – en reproductie werden bepaald op 26 dagen na inoculatie.

Er werden geen significante verschillen geobserveerd tussen het aantal nematoden dat de vier *Musa* genotypes gepenetreerd had op 4 en 8 dagen na inoculatie. Acht dagen na inoculatie werden eieren geobserveerd in Grande Naine maar niet in de drie resistente *Musa* genotypes. Het aantal eieren in de wortels van het resistente genotype Long Tavoy op 12 dagen was significant lager dan dat in Grande Naine. Het aantal eieren, mannetjes, vrouwtjes, het aantal volwassen nematoden en juvenielen en de finale populatiedensiteit waren significant hoger in het gevoelige genotype Grande Naine in vergelijking met de drie *R. similis* resistente *Musa* genotypes op 26 dagen na inoculatie. De post-infectionele nematodenontwikkeling en reproductie waren ernstig verhinderd in de resistente *Musa* genotypes. Het lijkt er dus op dat het resistentiemechanisme tegen *R. similis* in de onderzochte *Musa* genotypes geïnduceerd wordt na nematodenpenetratie en dat vooraf gevormde gastheerresistentiefactoren geen grote rol spelen in nematodenattractie, migratie naar de wortels, en wortelpenetratie.

Het tweede specifieke hoofddoel van deze studie was de identificatie van plantenchemicaliën in de resistente *Musa* genotypes die betrokken zijn bij de resistentie tegen *R. similis.* Een preliminaire studie werd uitgevoerd om de rol van lignine en fenolen in de resistentie van de drie *R. similis*-resistente *Musa* genotypes te bepalen, in vergelijking met Grande Naine.

De niet-geïnfecteerde planten van alle *Musa* genotypes waarvan stalen werden genomen op 6 weken na inoculatie vertoonden intense lignificatie in hun vaatbundels in vergelijking met de planten waarvan stalen werden genomen op 3 weken na infectie. Dit toonde aan dat de oudere wortels meer gelignificeerd waren in hun secundaire celwanden dan de jongere wortels. Nematodeninfectie verhoogde significant het ligninegehalte van Yangambi km5 wortels 6 weken na inoculatie. In alle vier *Musa* genotypes startte de lignificatie in de endodermis, om vervolgens uit te breiden naar de perifere weefsels van de vaatbundels met in het bijzonder de xyleemwanden en de parenchymcellen rond het xyleem en uiteindelijk naar het vasculaire parenchym (sclerenchym) in het centrum. De cortex en aerenchymcellen waren slechts beperkt gelignificeerd. Uitgebreide lignificatie is niet gerelateerd met de cortexcellen die direct betrokken zijn bij de defensie tegen *R. similis*. Dit toont aan dat de verhoogde lignificatie slechts een algemene defensierespons is ter bescherming van de vaatbundels en dient om schade aan de plant te verminderen. Lignificatie lijkt dus meer geassocieerd met tolerantie van de plant voor *R. similis* door schade te voorkomen, dan met resistentie tegen nematodenontwikkeling en reproductie. Histochemische lokalisatie van het totale fenolgehalte door kleuring met toluidineblauw toonde geen voorgevormde fenolische cellen in de cortex van de

R. similis-resistente en gevoelige *Musa* genotypes. Necrotische cellen geïnfecteerd door nematoden bevatten voornamelijk fenolische componenten. De Folin-Ciocalteu test toonde aan dat de nematodeninfectie verantwoordelijk was voor een verdubbeling van het totale fenolengehalte in alle *Musa* genotypes op 3 weken na infectie, en in de resistente genotypes op 6 weken na infectie. De verhoogde fenolsynthese kan het gevolg zijn van de biosynthese of accumulatie van secundaire metabolieten zoals fytoalexines in de nematodeninfectieplaatsen.

In het laatste deel van de karakterisatie van de nematodenresistentie in *Musa* genotypes, werd een combinatie van analytische technieken gebruikt om de secundaire metabolieten te identificeren die geïnduceerd werden in de nematodeninfectieplaatsen, namelijk "high performance liquid chromatography" (HPLC), "Proton nuclear magnetic resonance spectroscopy" (^{1}H NMR) en "ultra performance liquid chromatography coupled with mass spectrometry" (UPLC-MS). De secundaire metabolieten werden gelokaliseerd om hun distributie te bestuderen met behulp van "matrix-free laser desorption/ionisation mass spectrometric imaging" (LDI-MSI) technieken.

De HPLC en ^{1}H NMR analyses identificeerden op structurele manier negen fytoalexines van het fenylfenalenone-type, en de UPLC-MS analyse identificeerde nog een additioneel fytoalexine van het fenylfenalenone-type in het *R. similis*-geïnfecteerd necrotisch weefsel van bananenwortels. De resultaten leveren het duidelijke bewijs voor de inductie van secundaire metabolieten van het fenylfenalenone-type in *Musa* spp. als reactie op een *R. similis* infectie. Deze componenten werden niet teruggevonden in niet-geïnfecteerde controlewortels. De aanwezigheid van de fenylfenalenones was uitsluitend gelokaliseerd in de nematoden-geïnfecteerde necrotische regio's van de bananenwortels. Observatie van de lesies toonde ook aan dat de lesies in Yangambi km5 klein zijn, niet-continu en niet uitbreidend tot hypersensitieve lesies. In Grande Naine echter waren de lesies groot, tunnelvormig en bedekten zij grotere delen van de geïnfecteerde wortels. Anigorufone werd geïdentificeerd als meest abundante secundaire metaboliet van het fenylfenalenone-type met aanwezigheid in de necrotische lesies van *R. similis*-resistente en gevoelige cultivars. Maar de concentratie van anigorufone per gebiedseenheid was veel hoger in Yangambi km5 in vergelijking met Grande Naine. De hogere concentratie van anigorufone en andere fytoalexines van het fenylfenalenone-type in enkele cellen van het *R. similis*-resistente cultivar kan een meer toxische omgeving creëren voor de nematode dan in Grande Naine.

De anti-nematode eigenschappen van de geïdentificeerde fenylfenalenones werden bestudeerd door middel van *in vitro* biotesten voor inhibitie van nematodenbeweeglijkheid en letaliteit. Elf van de dertien geteste fenylfenalenones hadden een inhiberend effect op de beweeglijkheid van *R. similis*. Deze resultaten vormen het eerste bewijs voor de anti-nematode eigenschappen van fenylfenalenones.

Anigorufone, 4-hydroxy-2-methoxy-9-phenylphenalenone en isoanigorufone waren opmerkelijk krachtige anti-nematode componenten en veroorzaakten quiescentie in meer dan 75% van de *R. similis* juvenielen en volwassenen. Hydroxyanigorufone en monohydroxy-anigorootin hadden ook een sterk inhiberend effect op *R. similis* beweeglijkheid na 24h incubatie, en het effect was ofwel stabiel ofwel verhoogde het in de tijd. Anigorufone was de meeste krachtige anti-nematode component die getest werd. De vereiste concentratie van anigorufone om 50% quiescentie te veroorzaken (IC_{50}) bedroeg slechts 23ppm na 72h incubatie. Bij hogere concentraties van anigorufone, zoals 50, 100 en 150ppm, namen nematoden grote hoeveelheden van de component op in hun lichaam, wat leidde tot hun dood. Accumulatie van het opgenomen anigorufone werd gevonden in het darmkanaal van de nematoden van alle levensstadia. Uitgebreide biotesten waarbij gebruik gemaakt wordt van verschillende combinaties van de geïdentificeerde componenten zullen in de toekomst onze kennis vergroten van synergie of antagonisme tussen deze componenten.

Verdere studies zouden de mogelijkheden kunnen onderzoeken om de cellulaire concentratie van anigorufone en andere phenylphenalenones te verhogen en hun lokalisatie te verbeteren in de wortels van *R. similis*-gevoelige bananencultivars die commercieel succesvol zijn, zoals Grande Naine.

CHAPTER 1

GENERAL INTRODUCTION

1.1. OBJECTIVES OF THE STUDY AND OUTLINE OF THE THESIS

Nematodes are the second-most important limiting biotic factor of banana[1] production after the black Sigatoka leaf streak disease caused by the fungus *Mycosphaerella fijiensis* (Gowen *et al*., 2005). The burrowing nematode, *Radopholus similis* (Cobb, 1893) Thorne, 1949 is considered as the most damaging nematode species in commercial banana plantations (Sarah, 2000). Plant-parasitic nematodes are notoriously challenging pests to control. Complete eradication of *R. similis* in infested banana plantations requires a 5-years-crop free fallow, as the nematodes can survive on alternative weeds (EPPO, 2008).

Nematicides have been intensively used to control plant-parasitic nematodes. However, many effective nematicides have been withdrawn from the market due to their adverse effects on the environment, non-target organisms and the accumulation of toxic residues in food chain. The use of resistant cultivars is an efficient and economical alternative approach for controlling nematode populations (Fuller *et al*., 2008; Zasada *et al*., 2010). *Musa* cultivars resistant to *R. similis* have been identified. However, the commercially successful Cavendish-type dessert bananas are highly susceptible to *R. similis*. Unraveling the mode(s) of action or gaining in-depth knowledge of the precise mechanism(s) of host resistance to a nematode may provide information that can be used either to improve the efficacy of the use of the resistance or to assist in the faster selection or breeding of resistant cultivars.

The overall objective of our study was to characterise the mechanism(s) of resistance to *R. similis* in *Musa* spp. To achieve this objective, the well-known *R. similis*-resistant *Musa* cultivar Yangambi km5 along with two recently identified *Musa* resistance sources, Long Tavoy and Saba (Dochez *et al*., 2006), were studied.

The *R. similis* resistance of the recently identified new resistant *Musa* genotypes was first verified and compared with the resistance of Yangambi km5 under greenhouse conditions. The host response of these *Musa* genotypes to *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 infection was also evaluated to examine if the *R. similis*-resistant genotypes are also resistant to *M. incognita*, a root-knot nematode species which is increasingly reported as a major nematode associated with bananas in the absence of *R.*

[1] Bananas and plantains will alternately be referred to either as banana(s) or *Musa* spp., and where used as an adjective, they may be referred to simply as *Musa* (*i.e. Musa* cultivars).

similis in certain geographical areas (Chapter 2). In this thesis, the mechanisms of *Radopholus similis* resistance in *Musa* genotypes are studied in the further chapters. If a genotype is found to be resistant to both *Radopholus similis* and *Meloiogyne incognita*, the same mechanisms could operate against both nematodes. This can be highly interesting for future studies.

The first major specific objective of our study was to find out at which phase of the nematode-plant interactions (*i.e.* pre- or post-infection), the resistance to *R. similis* in the four *Musa* genotypes was active. To achieve this specific objective, an autotrophic *in vitro* model system was developed (Chapter 3) and greenhouse experiments were conducted to compare *R. similis* attraction, migration, penetration, development and reproduction in Long Tavoy, Saba, Yangambi km5 and in the well-known *R. similis*-susceptible *Musa* cultivar Grande Naine (Chapter 4).

The second major specific objective of our study was to identify the phytochemicals involved in the *R. similis* resistance of the *Musa* genotypes. A preliminary phytochemical profiling was performed to localise and quantify lignin and total phenols in the *Musa* genotypes (Chapter 5). A more detailed phytochemical profiling of the roots of Yangambi km5 and Grande Naine was carried out to precisely identify the secondary metabolites synthesized in the *R. similis*-infected cells. A combination of analytical techniques namely high performance liquid chromatography (HPLC), proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and ultra performance liquid chromatography coupled with mass spectrometry (UPLC-MS) and matrix-free laser desorption/ionisation mass spectrometric imaging (LDI-MSI) technique was used for the identification and localisation of phenylphenalenone-type phytoalexins present in the *Musa* roots (Chapter 6.1). The anti-nematode properties of the phenylphenalenones found in the *Musa* roots were assessed by *in vitro* bio-assays on nematode motility inhibition and nematode mortality (Chapter 6.2).

Finally, general conclusions and perspectives for further study are presented in Chapter 7. The outline of our study is shown in Figure 1.1.

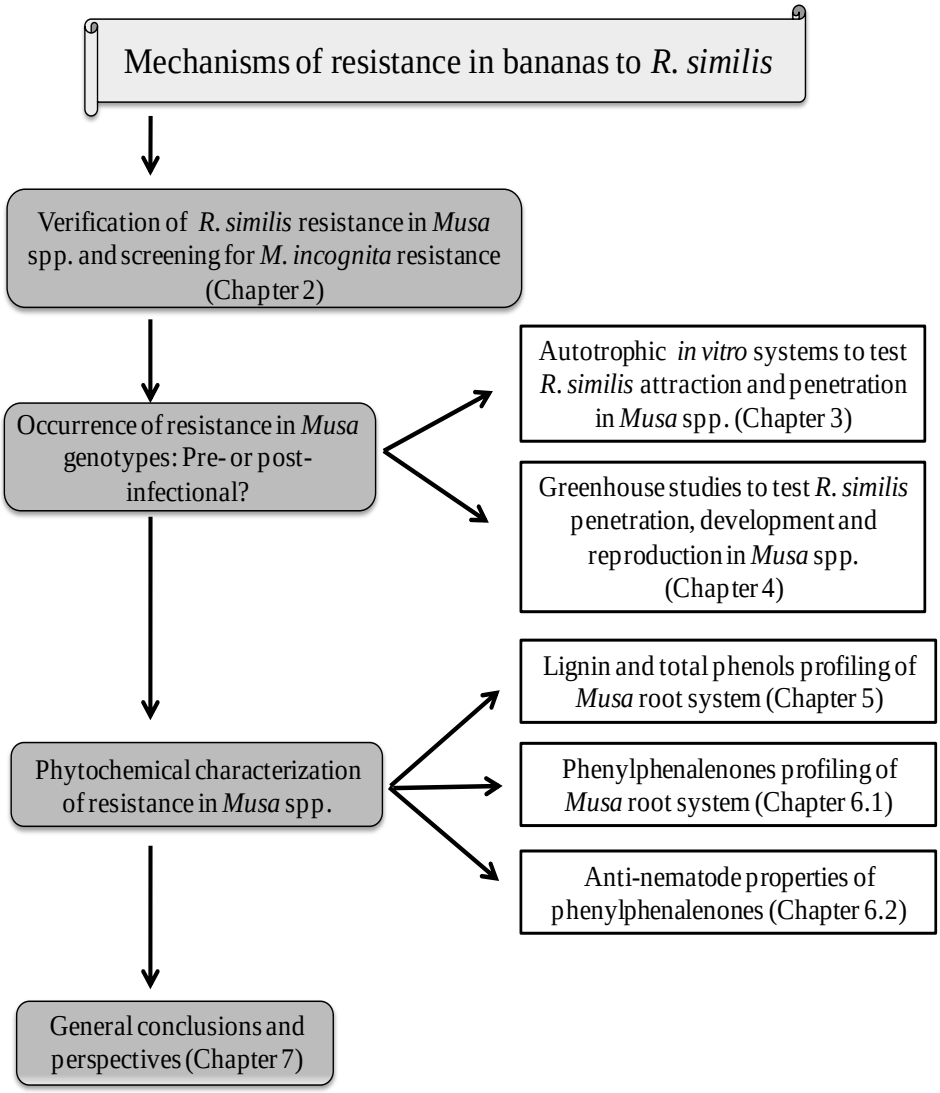


Figure 1.1: Flow chart representing the outline of our study.

1.2. *MUSA* SPP. - SOCIAL, CULTURAL AND ECONOMIC IMPORTANCE

Bananas and plantains, (*Musa* spp.), the largest herbaceous flowering plants of the world, originated in Southeast Asia. Their natural distribution is mainly restricted to the humid and sub-humid tropics of Asia, Africa and the Americas (Heslop-Harrison & Schwarzacher, 2007). Bananas are a highly valued commodity due to their social, cultural and economic importance. In the Indian sub-continent, bananas are considered as divine plants as almost all plant parts are used for human consumption. Apart from the fruits, the pseudostem and the male flower buds are consumed as vegetables. Uniquely, banana leaves possess cultural importance in many Asian countries as they are used to serve food during special occasions (Fig. 1.2).

Figure 1.2: A) Food served on a banana leaf in southern India. B) A small shop in a local market showing a high diversity of bananas for sale. Souce of B: Google.

Bananas are economically important crops in all tropical regions. World banana production in 2009 was 97.38 million tons produced on 4.92 million ha in more than 130 countries. The mean productivity was 19.78 tons/ha. Approximately 18% of the world banana production is exported, mainly to the non-producing northern hemisphere (FAOSTAT, 2010). In spite of the high biodiversity of bananas (Fig. 1.2), the export trade is almost exclusively limited to the Cavendish-type dessert bananas. Export is dominated by a few countries such as Costa Rica, Ecuador, Colombia and the Philippines. In the majority of the other producing countries, most of the production is consumed locally. As a chief source of food, bananas provide a well-balanced diet, thus contributing to the food security of more than 400 million people in the developing world (Singh, 2002).

The production of bananas is often restricted by diseases and pests. Nematodes are ranked as the second-most important limiting biotic factor of banana production after the black Sigatoka leaf streak disease caused by the fungus *M. fijiensis* (Gowen *et al*., 2005).

1.3. PLANT-PARASITIC NEMATODES

Nematodes are unsegmented, thread-like worms. Their natural habitats include terrestrial and aquatic ecosystems ranging from mountains to oceans. However, for these animals it is crucial that their body is always surrounded by a thin film of water for their mobility and survival (Perry, 1996).

Nematodes are highly adaptive and diverse. They are the most numerous metazoans on earth: four out of every five animals are nematodes (Bird & Kaloshian, 2003). Recently, a nematode species (*Halicephalobus mephisto*) has been found in the Earth's terrestrial deep subsurface, expanding the known metazoan biosphere (Borgonie *et al*., 2011). Nematodes are either free-living or parasites of plants and animals. Some plant-parasitic nematode species can damage the aboveground plant parts but the majority of the plant-parasitic nematode species are root pathogens. Most of the plant-parasitic nematodes are smaller than 1 mm.

Based on their life cycle and feeding habit, plant-parasitic nematodes can be classified as 1) ectoparasites that live completely outside the plant and feed on the outer cell layers of the roots with the aid of a stylet, 2) semi-endoparasites that enter the roots partially and feed on the inner cell layers of the roots while a part of the nematode's body remains outside in the soil, 3) endoparasites that penetrate the root completely and feed on the cortical and/or vascular cells of the roots. Endoparasitic nematodes can be further sub-divided into two types: a) sedentary endoparasitic nematodes of which the females become sessile after inducing specialized feeding cells on which they exclusively feed and b) migratory endoparasitic nematodes of which the juveniles and adults remain mobile and continuously feed on the cortical cells of the roots as they migrate inside the roots (Speijer & De Waele, 1997; Siddiqi, 2000).

1.3.1. BANANA NEMATODES

Although approximately 150 different nematode species were found associated with *Musa* spp., the following nematode species are considered the most important nematodes of this crop (Speijer & De Waele, 1997; Gowen *et al*., 2005): the burrowing nematode *R. similis*, the root-lesion nematodes

Pratylenchus goodeyi Sher & Allen, 1953 *Pratylenchus coffeae* (Zimmermann, 1898) Filipjev & Schuurmans Stekhoven, 1941 and the spiral nematode *Helicotylenchus multicinctus* (Cobb, 1893) Golden, 1956. Root-knot nematodes (*Meloidogyne* spp.) have also been often found associated with *Musa* spp. (Gowen *et al*., 2005). For our study, we have used *R. similis* as this species is considered the most important nematode species of banana worldwide.

1.3.1.1. The burrowing nematode *Radopholus similis*

More than a century ago, *R. similis* was first isolated from banana roots in the Fiji Islands (Cobb, 1893). As a migratory endoparasitic nematode, all life stages of *R. similis* remain mobile and are infective. *Radopholus similis* can be found worldwide on bananas in all tropical and subtropical regions with a mean annual temperature of 24-32 °C. The optimum temperature for its reproduction is about 30 °C. Survival of *R. similis* in temperate regions is limited only to greenhouses as the reproduction stops below 16-17 °C (as well as above 33 °C) (Sarah *et al*., 1996). A diagram of the *Radopholus similis* is provided in the annexes.

1.3.1.2. *Radopholus similis* host range and economical importance

Although the best known hosts of *R. similis* are bananas, black pepper and citrus, this nematode species has been found associated with hundreds of different plants (Holdeman, 1986) including arecanut, avocado, coconut, coffee, ginger, palm, sugarcane, tea, turmeric and ornamentals belonging to the families Araceae and Marantaceae (Moens & Perry, 2009). *Radopholus similis* is grouped among the 10 most damaging plant-parasitic nematode species around the world (EPPO, 2008). It causes severe yield losses by causing the "toppling" disease on bananas, "yellows" disease on black pepper and "spreading decline" on citrus.

1.3.1.3. *Radopholus similis* as a major root pathogen of bananas

Radopholus similis is considered as the most damaging nematode species in intensive commercial banana plantations, especially of Cavendish-type dessert bananas (Sarah, 2000). This nematode continuously feeds on the cortical cells. Collapse of the root cell walls ultimately leads to cavities and tunnels due to necrosis of the damaged root cells. This damage to the roots reduces water and nutrient uptake and translocation, causing impaired plant

growth and development, a prolongation of the vegetative stage and thus crop cycle, loss of bunch weight and poor root anchorage. Severe root damage may cause the whole plant to topple under strong windy conditions (Sarah, 2000; Gowen *et al*., 2005).

The average yield loss in bananas by *R. similis* was estimated as 20% (Sasser & Freckman, 1987). However, yield losses of bananas up to 75% over a 3-years cropping period caused by *R. similis* have been reported (Sarah, 1989). Nematicidal treatment in *R. similis*-infested plots doubled plantain yield compared to untreated plots in which about 50% of the plants toppled over a 2-years cropping period (Fogain, 2000a). Together with *H. multicinctus*, *R. similis* caused about 71% of plantains to die at flowering of the first ratoon crop (Coyne *et al*., 2005).

1.3.1.4. Control and management of *Radopholus similis* on bananas

Conventionally, large amounts of nematicides were used in the commercial banana plantations to limit yield losses caused by *R. similis*. However, because of environmental concerns, several efficient fumigant-nematicides such as DD (dichloropropane-dichloropropene), EDB (ethylene dibromide), DBCP (1,2-dibromo-3-chloropropane) and methyl bromide which were highly used until the 1980s have been phased out and withdrawn from the market (Quénéhervé, 2009). As a result, chemical nematode control currently depends upon some organophosphate and carbamate nematicides such as Aldicarb (Temik), Etoprop (Mocap), Fenamifos (Nemacur), Furadan (carbofuran) and Terbufos (Counter) which are applied 2-4 times a year (Villanueva, 2003; Quénéhervé, 2009). However, the intensive application of these organophosphate and carbamate nematicides continues to pose a threat to the environment and the health of agricultural workers (Atkinson *et al*., 2004). Moreover, strict regulations are exercised in many countries on the use of these nematicides that are still in use (Villanueva, 2003; Quénéhervé, 2009). Cultural practices such as fallowing, crop rotation, *etc*. are also used to control nematodes but are not applicable in banana fields. Also, they are often not adequate enough as a sole practice to control nematodes (Lilley *et al*., 2007). Complete eradication of *R. similis* from infested banana plantations requires a 5-years-crop free fallow as the nematode can survive on alternative weeds (EPPO, 2008). Using resistant cultivars to manage nematode populations in severely infested fields offers an efficient and economical alternative nematode management strategy (Fuller *et al*., 2008; Zasada *et al*., 2010).

1.4. HOST PLANT RESISTANCE IN *MUSA* SPP. TO *RADOPHOLUS SIMILIS*

In nematology, resistance is defined as the ability of a host plant to prevent or suppress nematode multiplication while in a susceptible host plant nematodes can multiply. Tolerance is independent of resistance and is defined as the ability of a host plant to suffer little damage even when quite heavily infected with nematodes while a sensitive host plant will suffer much injury even when relatively lightly infected with nematodes (Bos & Parleviet, 1995). This terminology is illustrated in Figure 1.3.

Using natural host plant resistance for the management of plant-parasitic nematodes offers a sustainable, ecologically-friendly and cost-effective alternative to the use of pesticides (Roberts, 1992; Fuller *et al*., 2008). However, most known resistance sources are specific only to certain nematode species. Also, resistance sources to the predominant nematode species have not been identified yet in many agricultural crops (Young, 1992; Atkinson *et al*., 2003; Lilley *et al*., 2007).

In bananas, numerous studies were carried out around the world to identify *Musa* genotypes with natural resistance to *R. similis* (Quénéhervé *et al*., 2008b). Wehunt *et al*. (1978) were the first to report that *Musa* cultivars belonging to the subgroup Pisang Jari Buaya (AA genome) were resistant to *R. similis*. This discovery intensified the search for additional resistant sources (see for instance Price, 1994; Fogain & Gowen, 1998; Stoffelen *et al*., 2000; Viaene *et al*., 2003; Dochez *et al*., 2005, 2006; Quénéhervé *et al*., 2008a, 2008b, Dizon *et al*., 2010; Herradura *et al*., 2011). Yangambi km5 (AAA) was another *R. similis*-resistant source that was studied elaborately (Price, 1994; Fogain & Gowen, 1998).

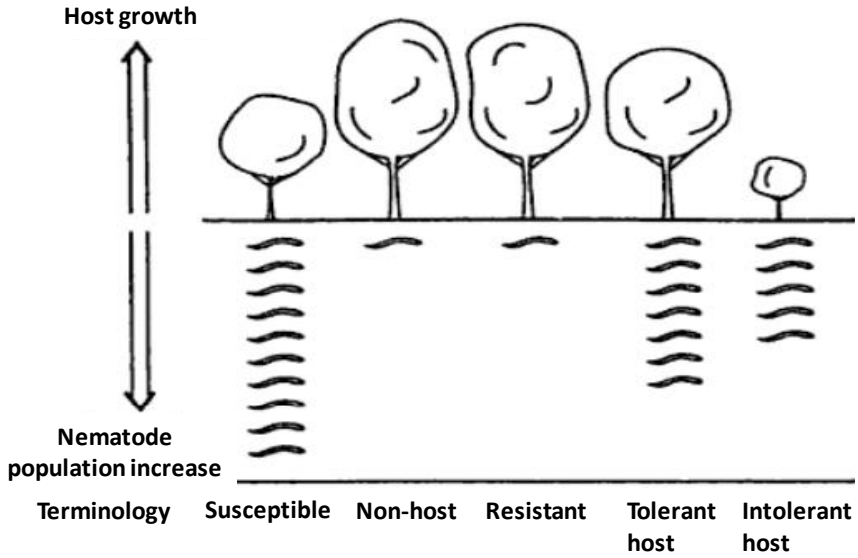


Figure 1.3: Host plant/pathogen relationship terminology used in nematology. Source: Roberts, 2002.

1.4.1. HOST-NEMATODE INTERACTIONS

An in-depth understanding of the interactions between a host plant and its pathogen(s) is crucial for the development of a successful management strategy. Plant infection by nematodes is a continuous and dynamic process. In the nematode infection cycle the following phases can be distinguished:

1) localisation of the host plant by the nematode (including migration by the nematode towards the host plant)
2) penetration and entry of the nematode in the host plant
3) nematode development
4) nematode reproduction.

The host-nematode interactions can be either successful or unsuccessful for the nematode. In the first case the plant is susceptible. In the second case the plant is resistant or a non-host.

1.4.1.1. Localisation of the host plant by the nematode

Plant-released cues are exploited by plant-parasitic nematodes to guide themselves towards the roots of their host plants. Plant roots release chemically-rich exudates and diffusates. Additionally, several other gradients existing in the rhizosphere, such as a CO_2 gradient, microbial density, redox potential or an electric field, can serve as guiding signals for nematodes to locate their host plants (Perry, 1996; Spence *et al*., 2008). Nematodes possess

sensory receptors in their head region. The pair of amphids is considered as a primary chemosensory receptor along with inner and outer labial sensilla which are considered as vital in nematode host finding and identification of suitable penetration sites (Perry, 1996). Host localisation or nematode attraction takes place over a distance of several centimeters. Nematodes sense the long-distance, short-distance and local attractants operating at several centimeters, in the close proximity of roots and at the direct contact of the host plant, respectively (Spence *et al*., 2008).

Long-distance cues are, in general, non-specific plant signals such as a CO_2 gradient (Robinson, 1995). Short-distance cues are usually host-specific. Nematodes with a broader host range, such as *Meloidogyne* spp., seem to respond to short-distance but non-specific plant signals (Robinson, 2002). Nematode host finding can be inhibited by blocking either the release of host-signaling molecules or the nematode's chemoreceptors (Zuckerman, 1983). Lectins were shown to inhibit the attraction of *R. similis* towards the banana roots *in vitro* (Wuyts *et al*., 2003). However, inconsistent effects were observed as treating *R. similis* with lectins resulted in enhanced root penetration by the nematode (Kaplan & Davis, 1991). Certain nematicides, such as aldicarb, impair nematode orientation at low concentration without affecting nematode movement (Perry, 1996).

1.4.1.2. Penetration and entry of the nematode in the host plant

Once nematodes have found the host roots, they invade the roots using their specialised feeding apparatus, the stylet and pharyngeal gland secretions. The stylet is a thin, hollow, needle-like tube located at the anterior end of the nematodes. Nematodes attack the plant's epidermal cells by repeated stylet thrusts to pierce the plant cell wall. In addition to physical damage, they also produce pharyngeal gland secretions to dissolve the cell wall. *Radopholus similis* possesses three large pharyngeal glands secreting different enzymes including cell wall degrading enzymes such as endo-1,4-β-glucanases (Haegeman *et al*., 2008) and endoxylanase (Haegeman *et al*., 2009). These two enzymes aid *R. similis* in penetrating the host and in migrating inside the host as they degrade the major cell wall components cellulose and xylan, respectively. *Radopholus similis* is capable of invading the plant root at any place albeit that they seem to prefer root tips and tender roots (Sarah *et al*., 1996). In a successful penetration, nematodes breach the first-line physical and chemical barriers of the host plant and penetrate it. In an incompatible interaction, plants are able to block nematode penetration by early defence

responses such as cell wall strengthening by lignifications or pre-existing specialised cell layers, *etc*.

Biochemicals that may be involved in resistance of plants to nematodes are discussed later in this chapter, in the section 1.5.

1.4.1.3. Nematode development and reproduction in the host

Following penetration, *R. similis* migrates through the cortex both inter- and intra-cellularly and continuously feeds on the cortical cells by sucking and digesting the cytoplasm using enzymatic secretions. *Radopholus similis* keeps migrating to new cortical cells to feed on (Sarah *et al*., 1996; Gowen *et al*., 2005). Juveniles develop into further developmental stages while females start laying one to six eggs per day (Haegeman *et al*., 2010). Resistant plants prevent nematode development and reproduction through hypersensitive responses, anti-nematode toxins or by inhibiting nematode feeding.

1.4.2. POSSIBLE MECHANISMS OF PLANT RESISTANCE TO NEMATODES

Natural plant resistance mechanisms towards nematodes (and other pathogens) can be classified into a) preformed resistance mechanisms and b) induced resistance mechanisms (Fig. 1.4).

1.4.2.1. Preformed resistance mechanisms

Pre-existing (passive) structural features such as root thickness, waxiness of the cuticle, degree of secondary wall thickenings, vascular structure, *etc*., have been reported to contribute to plant resistance to pathogens including plant-parasitic nematodes (Hutcheson, 1998). For instance, coffee clones resistant to the root-lesion nematode *P. coffeae* possessed more hairy roots, thicker epidermal and endodermal cell walls, and higher total polyphenol contents than susceptible clones (Toruan-Mathius *et al*., 1995).

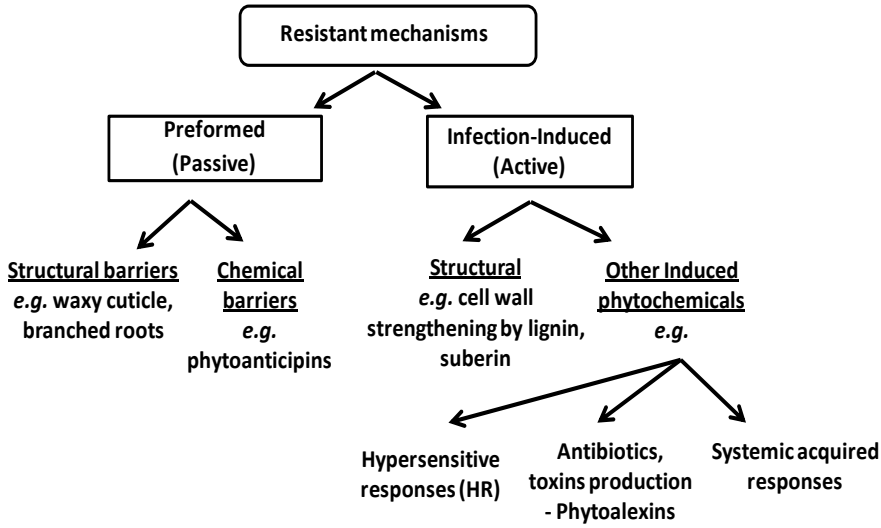


Figure 1.4: Possible mechanisms of plant resistance to nematodes. HR: hypersensitive response.

Constitutive antimicrobial secondary metabolites present in the plants may contribute to the preformed chemical mechanisms of nematode resistance. The polythienyl compounds extracted from *Tagetes* spp. are well documented as anti-nematode compounds. A phytochemical, thiophene α-terthienyl isolated from *T. erecta* plants was reported as toxic to the potato cyst nematode *Globodera rostochiensis*, the wheat seed gall nematode *Anguina tritici* and the stem and bulb nematode *Ditylenchus dipsaci*. In soil, the isothiocyanates and glucosinolates released by plants belonging to the family Brassicaceae are active against a variety of nematodes and insects (Chitwood, 2002)

1.4.2.2. Induced resistance mechanisms

Induced resistance mechanisms include active, energy-requiring responses of plants after infection by a pathogen. The triggering of these strong responses requires specific recognition of certain molecules from the invading pathogen which are collectively known as elicitors. Experiments have shown that in the absence of the pathogen, elicitors are capable of initiating the active plant defence response (Garcia-Brugger *et al*., 2006; Sanchez-Estrada *et al*., 2009). When resistant plants recognise matching elicitors, intracellular signal transduction pathways are activated resulting in an integrated series of plant responses leading to eventual neutralization of the invading pathogen (Fig. 1.5) (Dixon *et al*., 1994; Williamson & Hussey, 1996; Das *et al*., 2008; Fuller *et al*., 2008).

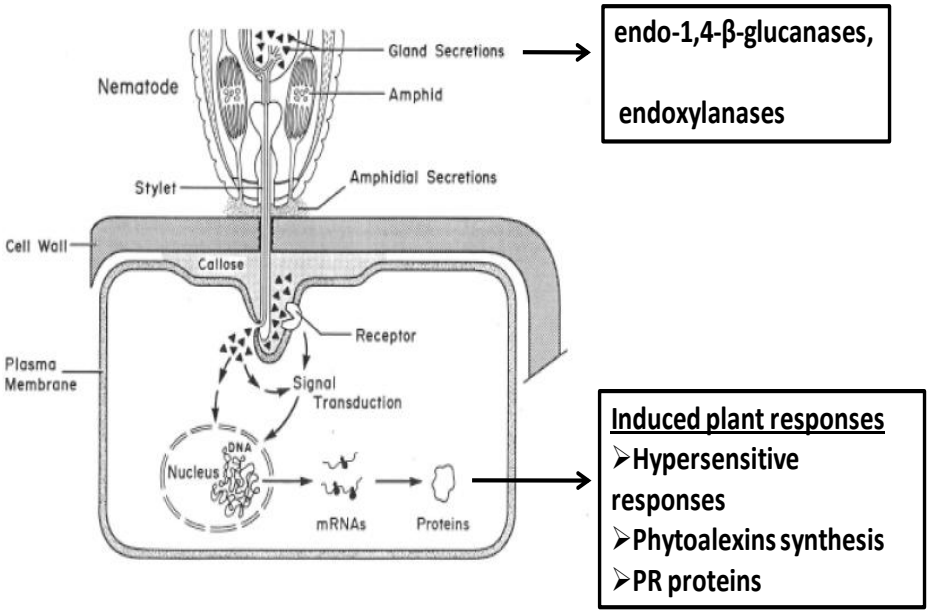


Figure 1.5: Host-nematode interaction and active plant responses. Nematode-released elicitors activate signal transduction pathways in the plant resulting in many induced plant responses to limit nematode development and reproduction. Source: Williamson and Hussey, 1996 (modified).

Key reactions of plants primarily include the hypersensitive response (HR) that consists of rapid death of plant cells in the immediate vicinity of the pathogen restricting the pathogen development. This is the primary response of the cell which comes in contact with the pathogenic organism (Gilchrist, 1998; Lam *et al*., 2001; Fuller *et al*., 2008).

Secondary responses are induced in the adjacent cells surrounding the initial infection site in response to diffusible signal molecules that are produced during the primary interaction. Secondary responses include the production and accumulation of toxic natural products known as phytoalexins and hydrolytic enzymes to antagonise the development of the pathogen (Hammerschmidt, 1999; Gheysen & Fenoll, 2002). Toxic secondary metabolites are deployed to site of pathogen challenge by vesicle-mediated trafficking (Field *et al*., 2006).

The third category of active plant defense responses is associated with systemically acquired resistance (SAR) that is hormonally induced throughout the plant (Hutcheson, 1998; Conrath, 2006). For instance nicotine is biosynthesised in root cells following insect attack or wounding. After its biosynthesis, the nicotine is translocated to the leaves of tobacco *via* the xylem

and accumulated in the leaves' vacuoles as a defensive toxin against herbivores (Yazaki *et al.*, 2008).

Possible biochemical mechanisms of resistance and the secondary metabolites involved in resistance in *Musa* spp. to *R. similis* are discussed later in this chapter, in the sections 1.5.3 and 1.5.4.

1.5. PLANT SECONDARY METABOLITES INVOLVED IN HOST PLANT RESISTANCE

Secondary metabolites are chemical compounds of metabolism that are not essential for photosynthesis, respiration, growth, development and reproduction of an organism but play a role in the interaction of the organism with its environment, ensuring the adaptation and survival of the organism in its ecological niche (Verpoorte, 2000). The importance of these natural products can be illustrated by the fact that approximately 25% of the known *Arabidopsis thaliana* genes are implicated in natural product synthesis and more than 170 natural products from seven major classes of compounds have now been reported in *A. thaliana* (Ben Field & Osbourn, 2006). Secondary metabolites are extremely diverse comprising more than 200,000 structures (Hartmann, 2007). Their classification is complex. Major groups of plant secondary metabolites are a) phenolics such as simple phenols, polyphenols, xanthones, phenylpropanoids, stilbenes and glycosides, b) isoprenoid-like compounds such as terpenes, steroids and its glycosides, c) alkaloids such as caffeine and nicotine, and d) amino acids such as alliin or canavanine (Dixon, 2001).

Originally, secondary metabolites were thought to be metabolic sinks. Their functions were largely unknown and hence ignored (Hartmann, 2007). However, these secondary metabolites are increasingly attracting the attention of scientists due to the discovery of their role in plants as protectants from pathogens, as attractants for pollinators and seed dispersers, as allelopathic agents and UV protectants. Besides, they also form valuable products such as dyes, fibres, glues, waxes, oils, drugs, perfumes, flavouring agents, *etc.* (Crozier *et al.*, 2006). Plants adapted different strategies to protect themselves from pathogens using secondary metabolites. Based on whether a plant accumulates secondary compounds as a preparation to face attacks by pathogens or secondary compounds are produced in response to attacks by pathogens, defence-related compounds are classified either as phytoanticipins or phytoalexins.

1.5.1. PHYTOANTICIPINS

Phytoanticipins are low molecular weight, antimicrobial compounds that are either present in plants before they are challenged by micro-organisms or they are produced after infection solely from pre-existing constituents (VanEtten *et al*., 1994). The distinction between a phytoanticipin and a phyoalexin is mainly based on when it is biosynthesized in the host, not on its chemical structure. In the cases where a constitutive metabolite is produced in larger amounts after infection, its status as a phytoanticipin or phytoalexin will depend on whether or not the constitutive concentrations were sufficient to cause antibiotic effects (Dixon, 2001).

Two different strategies have been adapted by plants in constitutive defense using phytoanticipins: a) constitutive accumulation of defense metabolites in specialised cells and b) constitutive accumulation of preformed defense compounds and respective enzymes in well-separated spatial compartments. Pathogenic attack could de-compartment them and release the active defense compound. For example, in *A. thaliana*, glucosinolates can be stored in the vacuoles of specialised cells known as S-cells (Koroleva *et al*., 2000) while the enzymes that activate them (myrosinases) are sequestered in myrosin cells (Rask *et al.*, 2000). Tissue damage caused by pathogen attack leads to breakdown of cell compartments followed by hydrolysis of glucosinolates to unstable aglucones by myrosinases. These unstable intermediates are then converted to toxic products such as isothiocyanates and nitriles (Ben Field & Osbourn, 2006). Baldridge *et al.* (1998) investigated the level of defense response isoflavonoids in alfalfa roots with different levels of resistance to the root-lesion nematode *Pratylenchus penetrans*. Constitutive levels of the phytoanticipin medicarpin were highest in the roots of the two most resistant plants.

1.5.2. PHYTOALEXINS

Müller (1961) defined phytoalexins as "compounds produced after infection under the influence of two metabolic systems, that of the host and that of the parasite, and inhibitory to the parasite." Since then, the term phytoalexin has undergone several conceptual changes becoming more inclusive of their production in response to physiological stress. Hence the new definition is "phytoalexins are low molecular weight antimicrobial/antibiotic compounds which are synthesized and accumulated in plants after exposure to pathogenic organisms or stress" (Paxton, 1980).

A long list of compounds can elicit the biosynthesis of phytoalexins (Kuć, 1995). This list includes the inorganic salts, oligoglucans, ethylene, fatty acids, polypeptides, some fungicides, low temperature and ultraviolet radiation. Also plant constituents released after injury or infection can function as elicitors.

A vast diversity of phytoalexins has been reported so far. More than 350 phytoalexins have been chemically characterized from approximately 30 plant families (Kuć, 1995). Due to their great diversity, there is no evident relationship between their chemical structure and their antibiotic property. Generally speaking, phytoalexins are found to be lipophilic, localised at and around infection sites (Kuć, 1995). However, each plant family possesses a certain unique set of secondary compounds equipping the plant to adapt to its own ecological niche (Hartmann, 2007). For example, sulphur-containing compounds such as glucosinolates are main phytoalexins in Brassicaceae (Morissey & Osbourn, 1999), isoflavonoids in the Leguminosae and sesquiterpenes in the Solanaceae (Dixon, 2001). But in contrast, the phenylpropanoid-type phytoalexins are present in many plant families such as Leguminosae, Solanaceae, Convolvulaceae, Umbelliferae, Gramineae, Cucurbitaceae and Musaceae (Dixon, 2001).

Phenolic compounds play an important role in the interactions of plants with their environment. They serve as phytoanticipins, phytoalexins, structural barriers, modulators of pathogenicity and activators of plant defense genes (Hammerschmidt, 2005). Two major groups of phenolic secondary metabolites, phenylphenalenones and phenylpropanoids, are reported to be involved in the defense response of *Musa* spp. to *R. similis*.

1.5.3. PHENYLPHENALENONES

1.5.3.1. Origin and natural occurrence

Phenylphenalenones are aromatic, naturally occurring secondary metabolites first isolated as natural pigments about 55 years ago. The term phenylphenalenone derives from the carbon skeleton of a phenalene-body, one keto group and an additional phenyl ring (Fig. 1.6).

The first phenalenone was isolated almost at the same time from a higher plant family, Haemodoraceae, and from fungi belonging to the Hyphomycetes and Discomycetes (Cooke & Segal, 1955b; Harman *et al*., 1955)

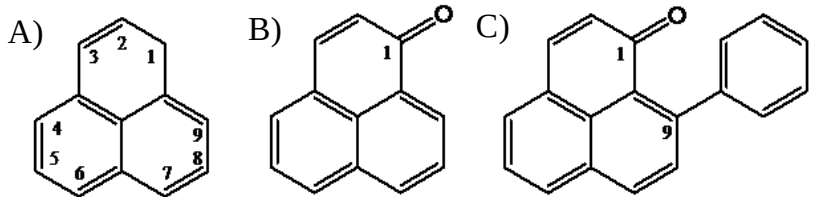


Figure 1.6: Structure of A) phenalen, B) phenalen-1-one and C) 9-phenylphenalen-1-one.

. However, the structures of the phenalenones produced by higher plants are significantly different from those produced by fungi i.e. the fungal phenalenones were lacking the additional phenyl group and are derived from an unrelated biosynthetic pathway (Fig. 1.7).

Haemocorin, a constituent of the rhizomes of *Haemodorum corymbosum*, was the first phenylphenalenone derivative to be characterized from plants (Cooke & Segal, 1955a,b). Following this description, many phenylphenalenones and related compounds were described from the same plant family, Haemodoraceae (Edwards & Weiss, 1974; Cooke & Dagley, 1979; Hölscher & Schneider, 1997).

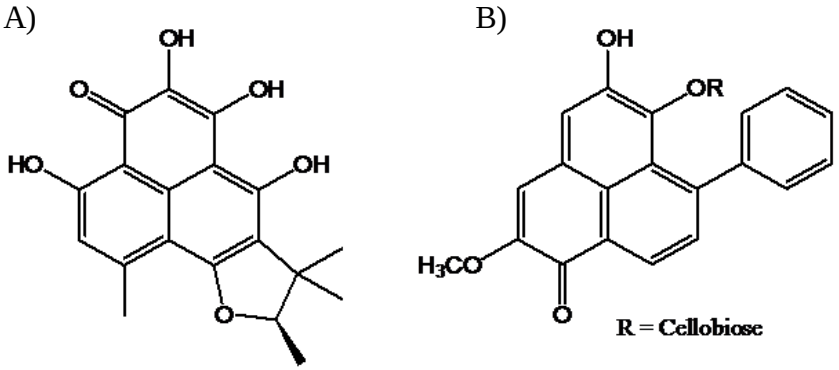


Figure 1.7: Structure of A) fungal phenalenone-atrovenetin and B) plant phenylphenalenone-haemocorin, first isolated from *Penicillium herquei* and *Haemodorum corymbosum*, respectively. Source: A: Harman *et al.*, 1955; B: Cooke and Segal, 1955b.

Only during the 1990s, phenylphenalenones were reported to be present in other plant families. But so far, their occurrence and distribution seem to be limited to some monocotyledonous families of the plant kingdom namely Haemodoraceae, Musaceae (Luis *et al.*, 1993), Pontederiaceae (Della Greca *et al.*, 1993) and Strelitziaceae (Hölscher & Schneider, 2000).

1.5.3.2. Biosynthesis of phenylphenalenones

Precursor incorporation studies have demonstrated that the structurally different fungal and plant phenalyphenalenones are also biosynthetically distinct from each other. The plant phenylphenalenone haemocorin is derived from phenylalanine and tyrosine (Fig. 1.8) (Thomas, 1971). In contrast, the known fungal phenalenones are biosynthesised via the polyketide pathway using acetate and malonate.

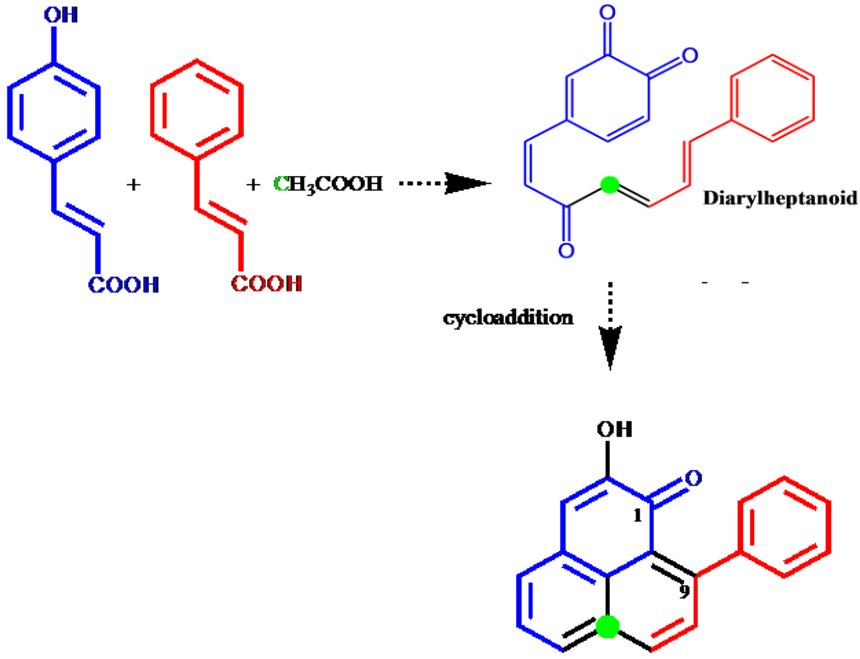


Figure 1.8: Biosynthetic pathway of 9-phenylphenalenone, anigorufone as hypothesized by Thomas (1971).

In plants, two aromatic amino acids, phenylalanine and tyrosine, the precursors of phenylphenalenones, are biosynthesised via the shikimic acid pathway (Edwards *et al*., 1972). These amino acids are converted into cinnamic acid by phenylalanine ammonia lyase (PAL) and coumaric acid by tyrosine ammonia lyase. Two units of phenylpropanoic acids, a variable incorporation of cinnamic acid, coumaric acid, caffeic acid and ferulic acid combine with a carbon of acetate or malonate to form a diarylheptanoid intermediate (Hölscher & Schneider, 1995a,b; Schmitt *et al*., 2000). The intermediate undergoes a structural modification and is then cyclised to form the C-19 skeleton of a phenylphenalenone (Schmitt & Schneider, 1999).

1.5.3.3. Biosynthesis of phenylphenalenones in Musaceae

Plants belonging to the Musaceae have been interesting models to study the biosynthesis of phenylphenalenones. When comparing the phenylphenalenones isolated from Musaceae and Haemodoraceae, many similar structural features and also considerable differences or specificity can be observed in phenylphenalenones belonging to the Musaceae. Specific differences to mention are the more common presence of 4-phenylphenalenones and abundance of naphthalic anhydrides in *Musa* spp. (Fig. 1.9). This stimulated discussions on the possibility of having a different biosynthetic pathway in *Musa* spp. (Luis *et al*., 1995; Kamo *et al*., 2000; Otálvaro, 2004).

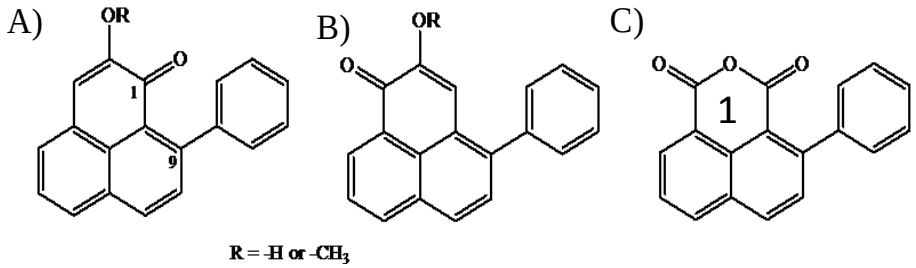


Figure 1.9: Structure of A) 9-phenylphenalenones commonly present in Haemodoraceae, Musaceae and other plants, B) 4-phenylphenalenones which are more common in Musaceae and C) Ring-1: Naphthalic anhydride type. Source: Otálvaro, 2004.

Based on the close taxonomical relationship between *Musa* spp. and *Curcuma longa*, Kamo *et al*. (2000) studied the possibility of *Musa* phenylphenalenones being biosynthesised along a pathway similar to that of curcumin biosynthesis. Precursors of curcumin have been reported to be derived from one phenylpropanoid and five malonyl-CoA moieties. However, from the results of their biosynthetic feeding experiments using [1-^{13}C] and [2-^{13}C]cinnamic acids and [2-^{13}C]malonate, Kamo *et al*. (2000) showed that two molecules of cinnamic acid and one of malonate were incorporated into each molecule of hydroxyanigorufone (a 9-phenylphenalenone) in *Musa acuminata*. Hence it was concluded that the phenylphenalenones biosynthesis in Musaceae is almost the same as in Haemodoraceae.

Some notable studies carried out on the biosynthesis of 4-phenylphenalenones of Musaceae hypothesised a common dihydroxylated or trihydroxylated intermediate compound but differed slightly in the further steps of biosynthesis (Luis *et al*., 1995; Kamo *et al*., 2000; Otálvaro, 2004). Kamo *et*

al. (2000) suggested that 9-phenylphenalenones were converted to 4-phenylphenalenones through a carbonyl transposition process. But, the other two postulations (Luis *et al*., 1995; Otálvaro, 2004) suggest a diversification of the biosynthetic pathway after the synthesis of the common intermediate compound leading to the formation of 4-phenylphenalenones. The presence of a 4-phenylphenalenone from *Anigozanthos preissii* (Hölscher & Schneider, 1997) and the isolation of a 5-methoxy-7-phenylphenalenone in *Dilatris* spp. (Hölscher & Schneider, 2007) recommend further studies to prove the hypotheses of unique presence of 4-phenylphenalenones in Musaceae.

1.5.3.4. Phenylphenalenones as phytoalexins and phytoanticipins

Haemodoraceae and Musaceae are the two major plant families in which a rich occurrence of phenylphenalenones has been found. Originally, phenylphenalenones were characterized as naturally occurring pigments in the Haemodoraceae in the genera *Anigozanthos*, *Conostylis*, *Dilatris*, *Haemodorum*, *Lachnanthes*, *Xiphidium* and *Wachendorfia* (Cooke & Edward, 1981; Dora *et al*., 1993; Hölscher & Schneider, 1997; Opitz & Schneider, 2002). Phenylphenalenones are constitutively present in the cells of plants belonging to these genera (Fig. 1.10).

In *Musa* spp., phenylphenalenones are produced as phytoalexins after the plant is being infected by pathogens (bacteria, fungi and nematodes) or chemical elicitors. Two phenylphenalenones, irenolone and hydroxyanigorufone, were first reported as phytoalexins in *Musa* spp. by Luis *et al*. (1993; 1995) following their biosynthesis in the leaves of the cultivar Grande Naine due to infection by *M. fijiensis*. Phenylphenalenones were also found to be produced and accumulated in banana fruits after infection by *Colletotrichum musae* (Kamo *et al*., 1998; 2001) and in rhizomes and roots after infection by *Fusarium oxysporum f. sp. cubense* (Luis *et al*., 1994, 1996, 1997).

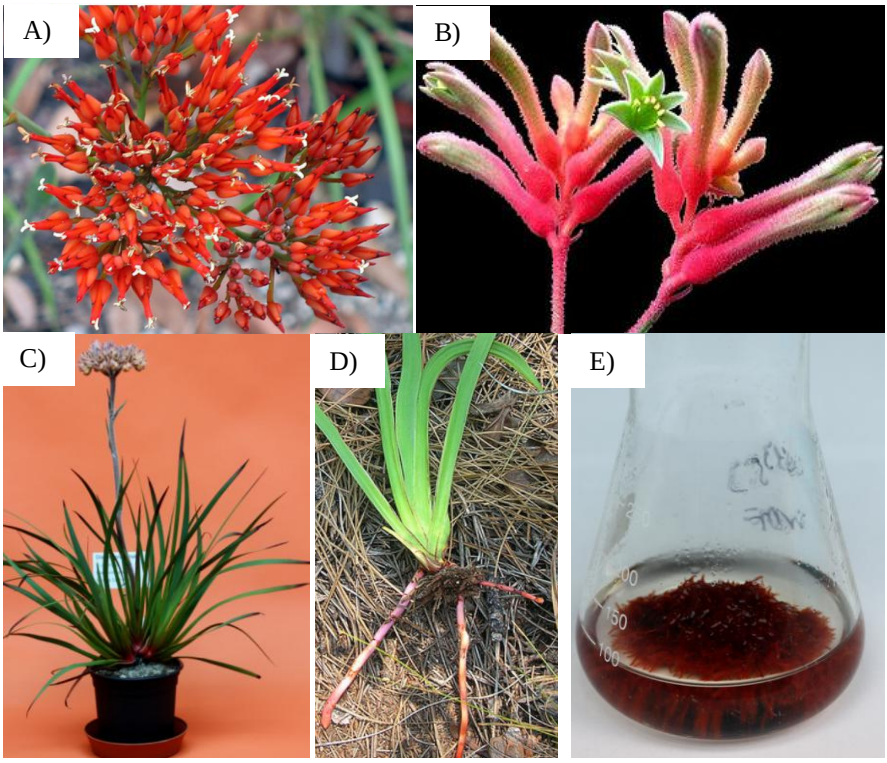


Figure 1.10: Inflorescence of A) *Heamodorum* sp., B) *Anigozanthus flavidus*, C) *Dilatris pillansii*, and blood red coloured roots of D) *Lachnanthes* sp. and E) *Wachendorfia thyrsiflora*. Source of C and E: Dr. Hölscher, Max-Planck-Institute for Chemical Ecology. A, B and D: Google.

Although most reports show that phenylphenalenones are biosynthesised in *Musa* spp. only after elicitation, rarely phenylphenalenones were also reported to be present in low concentrations in the healthy tissues of *Musa* spp. It was proposed that they function as phytoanticipins in these healthy tissues due to their occurrence in the *Musa* hybrid SH-3481 (Luis *et al*., 1999). This hybrid was reported earlier as resistant to *F. oxysporum f. sp. cubense* races 1 and 4, and to *M. fijiensis*. Concentration of constitutive hydroxyanigorufone in banana fruits increased greatly upon wounding without infection whereas all other phenylphenalenones were biosynthesised only after infection with conidial spores of *C. musae* (Kamo *et al*., 2000).

1.5.3.5. Phenylphenalenones as antibiotic compounds

Phenylphenalenones being synthesized as phytoalexins, their function as antimicrobial or antibiotic compounds, their mode of action and relationship between the structure of the compounds and their activity have become a major study field. Antibiotic properties of phenalenones were reported more than three decades ago. Phenalen-1-one was toxic to green algae, blue-green algae, and diatoms. With green algae and diatoms, this toxicity was strongly wavelength-dependent showing greater toxicity to green algae in white light (Winters *et al.*, 1977).

Antifungal properties of the phenylphenalenones, especially against plant pathogens, were reported. Phenylphenalenones isolated from *Musa* spp. inhibited the germination of *F. oxysporum f. sp. cubense* race 4 and moderately inhibited germination of *Alternaria* sp. (Luis *et al*., 1996, 1998). Kamo *et al*. (1998) studied the antifungal activity of 14 different phenylphenalenones and their derivatives by the thin layer chromatography (TLC) autobiography method. Six compounds namely anigorufone, irenolone, 2-hydroxy-4-(4´-methoxyphenyl)phenalen-1-one, (+) *cis* 2,3-dihydro-2,3-dihydroxy-4-(4´-hydroxyphenyl)phenalen-1-one, 2-(4´-hydroxyphenyl)-1-methoxycarbonyl-8-naphthalenecarboxylic acid and 2-(4´-hydroxyphenyl)-8-methoxycarbonyl-1-naphthalenecarboxylic acid, were shown to inhibit the spore germination of *C. musae*. These results lead the authors to conclude that a phenolic-hydroxyl group is essential to have this antifungal activity and 4-phenylphenalenones have stronger anti-fungal properties than 9-phenylphenalenones.

Systematic bioassays conducted using nine phenylphenalenones against *M. fijiensis*, the causal agent of black Sigatoka leaf streak disease on bananas, showed that the natural phytoalexins anigorufone, isoanigorufone, hydroxyanigorufone and irenolone inhibited mycelial growth and spore germination of this fungus albeit in very low intensity compared to the synthetic phenalenones, perinaphthenone. Perinaphthenone being an efficient singlet oxygen sensitiser, it was speculated that the high antifungal activity of this compound could have been due to the production of singlet oxygen among other possible complex biochemical interactions (Quiñones *et al*., 2000). Similarly, Lazzaro *et al*. (2004) attributed the enhanced antifungal activity of phenylphenalenones against *F. oxysporum* f. sp. *cubense* to the production of reactive oxygen species. Recently, two new phenylphenalenones, 2-hydroxy-1*H*-phenalen-1-one and 2-methoxy-1*H*-phenalen-1-one, isolated from the banana cultivar Yangambi km5, have shown inhibitory effect on the mycelial growth and spore germination of *M. fijiensis* (Otálvaro *et al*., 2007).

Antibacterial properties were also reported for these secondary metabolites. Populations of *Sarcina lutea* and *Escherichia coli* were inhibited by 2-methyl-9-(4´-hydroxyphenyl)-phenalen-1-one (Qin *et al*., 2006).

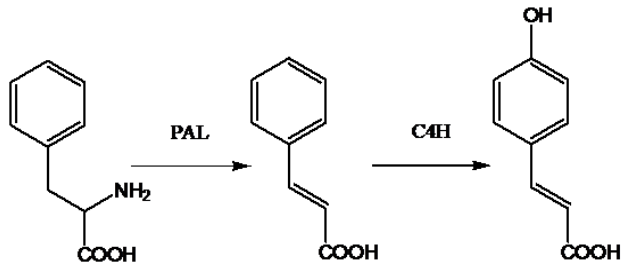
1.5.3.6. Phenylphenalenones as phytoalexins in plant-nematode interactions

Anigorufone was the first phenylphenalenone identified to be produced *de novo* in the roots of the banana cultivar Pisang Sipulu (group: Pisang Jari Buaya) in response to infection by *R. similis* (Binks *et al*., 1997). Since phenylphenalenones are produced as phytoalexins in response to nematode damage, it has been suggested that they play a significant role in the defense system of the plant (Luis *et al*., 1998). But, no information is available on the anti-nematode properties of these phenylphenalenones and on the role of phenylphenalenones in resistance or defense mechanism of *Musa* spp. to nematodes.

1.5.4. PHENYLPROPANOIDS

The phenylpropanoids are a diverse group of organic compounds that are synthesised from the amino acid phenylalanine through an enzymatic reaction mediated by the key enzyme phenylalanine ammonia lyase (PAL). Though structurally diverse, the name phenylpropanoids is derived from the core structure with six-carbon aromatic phenyl groups and three carbon propene tails of cinnamic acid (Fig. 1.11). Cinnamic acid is a common precursor of all phenylpropanoids and also the first product derived from phenylalanine by a nonoxidative de-amination in the core phenylpropanoid pathway (Dixon & Paiva, 1995). From the core pathway, specific pathways branch out leading to the synthesis of diverse compounds, such as coumarins, chlorogenic acid, isoflavonoids, flavonoids, suberin, lignin and other wall-bound phenolics.

As mentioned earlier, phenylpropaniods are one of the rare groups of compounds that are distributed throughout the plant kingdom. The phenylpropanoid pathway compounds have attracted the attention of plant physiologist, biochemists and pathologists due to their inducibility at the transcriptional level and their diverse functions. In plants, they function *inter alia* as flower pigments, UV protectants, antibiotic compounds (phytoalexins or anticipins) and signaling molecules in plant-microbe interactions. They are also vital for the structural integrity of plants as the plant cell walls are composed of polymeric phenylpropanoids such as lignin, lignans and suberins (Hahlbrock & Scheel, 1989).



Phenylalanine **Cinnamic acid** **Coumaric acid**

Figure 1.11: The core phenylpropanoid biosynthetic pathway. Source: Hahlbrock and Scheel (1989) (modified).

1.5.4.1. Phenylpropanoids and nematode resistance in *Musa* spp.

The biosynthesis of phenylpropanoids is induced by a variety of physical and biological stresses in plants including nematode infections (Fig. 1.12). The presence of phenolics and cell wall strengthening by lignifications and suberisation were often associated with host plant resistance to pathogens (Zacheo *et al*., 1997). Elevated syntheses of phenylpropanoids were correlated with plant resistance or defence response to nematodes in many crops. However, only a few studies involve the isolation of the compounds from the plants, their structural identification and confirmation of their anti-nematode properties (Chitwood, 2002).

In *Musa* spp., a few studies explored the possibility of the involvement of phenylpropanoids in the resistance to *R. similis*. Higher numbers of preformed phenolic cells were correlated with the resistance in the banana cultivars Yangambi km5 and Gros Michel. While in the banana cultivar Pisang Jari Buaya, a higher number of lignified cell walls was suggested to play a role in its resistance to nematodes (Fogain & Gowen, 1996).

Histochemical studies of the roots of Yangambi km5 revealed elevated levels of the constitutive compounds lignin, flavonoids, dopamine, caffeic esters and ferulic acids, suggesting their role in preventing *R. similis* penetration. Lignification and suberization in endodermal cells could stop *R. similis* from penetrating the stele (Valette *et al*., 1998). However, extensive lignifications were observed both in resistant as well as susceptible plants after *R. similis* infection (Wuyts *et al*., 2007). Following *R. similis* infection, early and extensive flavonoids accumulation occurred in cell walls adjacent to cavities formed by migrating nematodes (Valette *et al*., 1998).

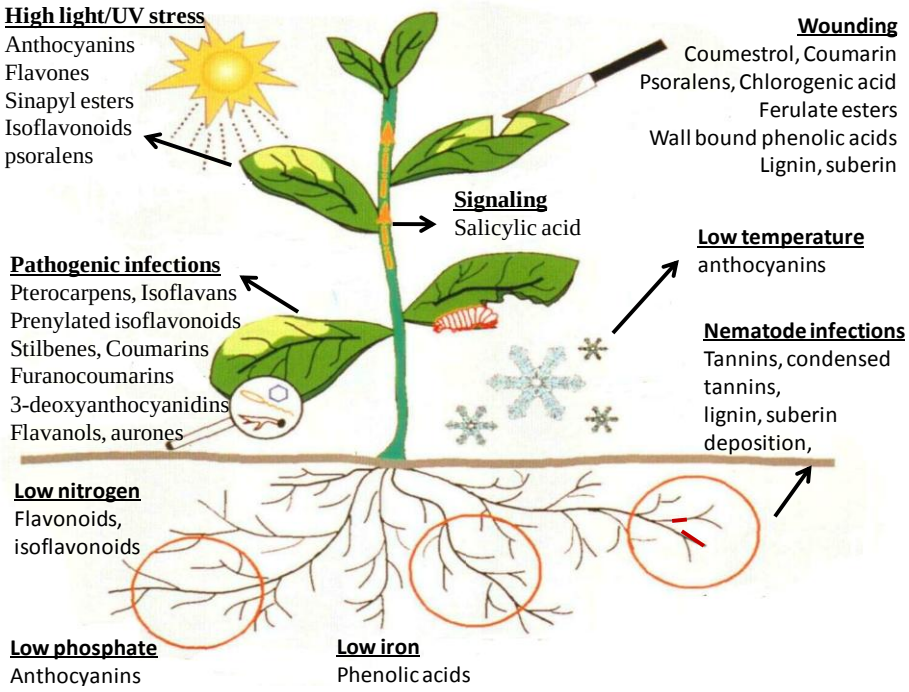


Figure 1.12: Phenylpropanoids and their accumulation in different plant parts in response to abiotic and biotic stresses. Source: Dixon and Paiva (1995) (modified).

Nematode infection increased the production of condensed tannins, including procyanidin and propelargonidins, in the *R. similis*-resistant banana cultivar Kunnen (Collingborn *et al*., 2000). However, no correlation between resistance and tannin contents was observed in a later study (Wuyts, 2006). Higher concentration of dopamine was observed in *R. similis*-resistant banana cultivars that could serve as substrate for polyphenol oxidation in necrotic tissues (Wuyts *et al*., 2007).

CHAPTER 2

IDENTIFICATION OF COMBINED RESISTANCE TO *RADOPHOLUS SIMILIS* AND *MELOIDOGYNE INCOGNITA* IN *MUSA* GERMPLASM[2]

[2] The results presented in this chapter were published as: SUGANTHAGUNTHALAM, D., ELSEN, A. AND DE WAELE, D. (2010). Identification of combined resistance to *Radopholus similis* and *Meloidogyne incognita* in *Musa* germplasm. *International Journal of Nematology* 20:19-24.

2.1. INTRODUCTION

Burrowing and root-knot nematodes are root pathogens that can cause serious yield losses to *Musa* spp. (Gowen *et al*., 2005). Worldwide, the burrowing nematode *Radopholus similis* (Cobb, 1893) Thorne, 1949 is considered as the most damaging nematode species in *Musa*-based cropping systems (Sarah *et al*., 1996; Gowen *et al*., 2005). However, under field conditions, usually several nematode species co-exist in the roots of bananas and plantains (De Waele & Elsen, 2007), and in the absence of *R. similis*, other, less important nematode species such as the root-knot nematode *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 can become more damaging to *Musa* spp. (De Waele & Davide, 1998; De Waele, 2000; Brentu *et al*., 2004; Cofcewicz *et al*., 2005; Van den Bergh *et al*., 2006).

The use of host plant resistance is considered as an economical and environmental-friendly nematode management strategy in the low-input cropping systems prevailing in developing countries (Roberts, 1992). This strategy also ensures maximal land use efficiency without changing the existing cropping systems (Atkinson *et al*., 2003). In *Musa*, a relatively high number of studies have been carried out to identify natural sources of resistance to *R. similis* resulting in the identification of some potential *R. similis*-resistant *Musa* genotypes (see for instance, Wehunt *et al*., 1978; Fogain & Gowen, 1998; Stoffelen *et al*., 2000; Viaene *et al*., 2003; Dochez *et al*., 2005, 2006; Quénéhervé *et al*., 2008a, 2008b; Herradura *et al*., 2011). However, the efficient use of natural sources of resistance to nematodes is limited due to several reasons (Roberts, 1992). A major limitation is that, in most cases, the resistance is restricted to one nematode species or a few pathotypes of the same nematode species. Planting of species-specific or pathotype-specific resistant cultivars under field conditions has resulted in a shift in nematode population composition causing less important nematode species to become more damaging and, sometimes, even a major problem (Young, 1992). To overcome this loss of efficiency, identification of plant genotypes with resistance to more than one nematode species or pathotype is necessary. Moreover, if these plant genotypes can be used in breeding programmes, their identification could lead to the development of cultivars with multiple nematode resistances.

The objective of this part of our study was to evaluate the host response of selected *Musa* genotypes to both *R. similis* and *M. incognita*. The *Musa* genotypes studied were selected based on their resistant host response to *R. similis* as reported by Dochez *et al*. (2006). For their experiments, Dochez *et al*. (2006) used sucker-derived planting material. Some studies have demonstrated

that tissue culture-derived *Musa* planting material can be more susceptible to *R. similis* compared to sucker-derived planting material (Blomme *et al*., 2004; Viaene *et al*., 2003) apparently due to their more fragile root system. In spite of this, tissue culture-derived plants are preferred as planting material for the initiation of new or the rejuvenation of existing commercial plantations and also for research purposes because of their homogeneity, availability throughout the year and because they are free of pathogens. In the first part of our study we re-confirmed the host response of the *R. similis*-resistant *Musa* genotypes reported by Dochez *et al*. (2006) using this time tissue culture-derived plants. In the second part of our study, we examined the host response of the same *Musa* genotypes to *M. incognita*, also using tissue culture-derived plants.

2.2. MATERIALS AND METHODS

2.2.1. EXPERIMENTAL SET-UP

Two experiments were carried out in the greenhouse at 27 and 20 °C day and night temperatures, respectively, 12 h photoperiod and 80% relative humidity. In the first experiment, seven *Musa* genotypes were examined for their host response to *R. similis*. This experiment was carried out in two separate batches. The host response of each genotype was compared to the host response of a susceptible (Grande Naine) and two resistant (Yangkambi km5, Pisang Jari Buaya) reference cultivars (Speijer & De Waele, 1997). In the second experiment, eight *Musa* genotypes were examined for their host response to *M. incognita*. In this experiment, Grande Naine was included as the susceptible reference cultivar (Speijer & De Waele, 1997). There was no resistant reference cultivar included as no *Musa* genotypes with resistance to *M. incognita* were known. In both experiments, all treatments were replicated eight times and a randomized block design was used. Some important characteristics of the selected genotypes and reference cultivars are listed in Table 2.1.

2.2.2. PLANTING MATERIAL

All *Musa* genotypes were initially obtained from the *Musa* germplasm collection maintained at the International Transit Centre (ITC), K.U.Leuven, Belgium. The plant material was proliferated, regenerated and rooted in test tubes on Murashige and Skoog medium including vitamins, 30 g/l sugar, 10 mg/l ascorbic acid and 2 g/l gelrite with pH 6.12 (Murashige & Skoog, 1962). Proliferation was obtained by adding 10^{-6} M indole-3-acetic acid (IAA) and 10^{-5} M 6-benzylaminopurine (BAP). For rooting, 0.05% (w/v) active charcoal was

added (Banerjee & De Langhe, 1985). No plant growth regulators were added. The plantlets were grown in growth chambers at 28 °C and 16 h photoperiod.

Table 2.1. Characteristics of selected *Musa* genotypes and the reference cultivars* evaluated for their host response to *Radopholus similis* and *Meloidogyne incognita*.

Genotype (subgroup)	ITC	Genome	Use	Host response	
				R. similis	*M. incognita*
Gia Hui (Pisang Awak)	1143	ABB	cooking	-	-
Kokopo	1243	AA	cooking/ dessert	-	-
Long Tavoy (Burbannica)	0283	AA	wild	-	-
Marau	0772	AAA (AAB)	cooking	-	-
Pisang Mas (Sucrier)	0653	AA	dessert	-	-
Pora Pora	0868	AA	cooking/ dessert	-	-
Saba	1138	ABB	cooking	-	-
Vudu Papau	0590	AA	dessert	-	-
Grande Naine* (Cavendish)	1256	AAA	dessert	S	S
Yangambi km5* (Ibota)	1123	AAA	dessert	R	-
Pisang Jari Buaya*	0312	AA	dessert	R	-

ITC: International Transit Centre, K.U.Leuven, Belgium.
S: susceptible; R: resistant.

Eight-weeks-old rooted tissue culture plantlets were planted in 1 l pots filled with sand and potting soil (2:1). The potted plants were maintained under greenhouse conditions (Fig. 2.1) and fertilized at 10 days intervals throughout the experiments.

Figure 2.1: Experimental set-up of the *Radopholus similis* host response experiment in the greenhouse.

2.2.3. NEMATODE INOCULUM AND INOCULATION

A population of *R. similis* originally isolated from banana roots in Uganda was used in the first experiment. This population was maintained and multiplied monoxenically on sterile carrot discs at 25±1 °C in the dark (Speijer & De Waele, 1997). The population from Uganda was characterized by a high reproductive fitness (Fallas *et al*., 1995). A *M. incognita* population originally isolated from banana roots in Malaysia was used in the second experiment. This population was maintained *in vitro* on *Ri* T-DNA-transformed tomato roots (Verdejo *et al*., 1988). This population was multiplied *in vivo* on tomato cv. Marmande roots under greenhouse conditions.

For the first experiment, *R. similis* was extracted from the carrot discs by the maceration-sieving technique (Speijer & De Waele, 1997). Eight weeks after planting, approximately 1,000 living vermiform nematodes were inoculated per plant by pipetting 4 ml of the nematode suspension into inoculation holes made in the soil near the plant root zone. For the second experiment, *M. incognita* was extracted from the *in vivo* tomato cv. Marmande roots by treating the roots with 1% NaOCl for 4 min to dissolve the gelatinous matrix of the egg masses followed by the maceration-sieving method (Speijer & De Waele, 1997; Hooper *et al*., 2005). Eight weeks after planting,

approximately 4,000 eggs and juveniles were inoculated per plant by pipetting 4 ml of the nematode suspension into inoculation holes made in the soil near the plant root zone.

2.2.4. EVALUATION OF THE HOST PLANT RESPONSE

Eight weeks after nematode inoculation (*i.e.* 16 weeks after planting), plants were uprooted and gently washed free of soil under running tap water. The fresh root and shoot weights, root damage and nematode reproduction were assessed.

Radopholus similis

Estimation of the percentage root necrosis caused by *R. similis* was based on the scoring of five randomly selected, 10-cm-long, longitudinally sliced functional primary roots (Speijer & De Waele, 1997). Then, the same roots were cut into 1-cm-long pieces, weighed and, if necessary, additional roots were sampled to make up 15 g of roots for nematode extraction by the maceration-sieving technique (Speijer & De Waele, 1997). The roots were macerated in a kitchen blender for three periods of 10 sec each separated by a 5 sec interval. The macerated suspension was passed through a series of 250, 100, 40 and 25 µm aperture sieves. Nematodes were collected from the 40 and 25 µm aperture sieves and pooled. Eggs, juveniles, females and males were counted separately from a 2 ml aliquot out of the homogenised suspension. The final nematode population densities were determined by calculating the sum of all developmental stages of the nematodes present in the total root system. The reproduction factor (Rf) was calculated as the final nematode population density divided by the inoculum. The host response of each genotype to *R. similis* was determined based on a comparison of the final nematode population density of each genotype with the reference cultivars as explained in Table 2.2.

Table 2.2. Identification of the host response of selected *Musa* genotypes to *Radopholus similis* based on a comparison with the host response of a susceptible (Grande Naine) and a resistant (Yangambi km5) reference cultivar.

Comparison of the final nematode population density		Host response
with Grande Naine	with Yangambi km5	
significantly lower	significantly lower	highly resistant
significantly lower	insignificant	resistant
significantly lower	significantly higher	partial resistant
insignificant	significantly higher	susceptible
significantly higher	significantly higher	highly susceptible
insignificant	insignificant	inconclusive

Significance was measured according to the Dunnett's test ($P \leq 0.05$).

Meloidogyne incognita

Roots were cut into 1- to 2-cm-long pieces; a sample of 5 g roots was taken at random and stained with phloxine B for visual observation of the egg masses (Hooper *et al*., 2005). The red stained egg masses were counted as egg-laying females using a stereoscopic microscope. After counting the egg-laying females, the same roots were used to extract the eggs and juveniles by solubilising the gelatinous matrix with 1% NaOCl followed by the maceration-sieving technique (Speijer & De Waele, 1997). The roots were macerated in a kitchen blender for three periods of 10 sec each separated by a 5 sec interval. The macerated suspension was passed through a series of 250, 100, 40 and 25 µm aperture sieves. Eggs and juveniles retained on the 40 and 25 µm aperture sieves were collected, pooled and counted in a 2 ml aliquot out of the homogenised suspension using a stereoscopic microscope. The final *M. incognita* population density (Pf) was calculated by the sum of eggs and (mostly) second-stage juveniles (J2) present in the whole root system. The reproduction factor (Rf) was calculated as the final number of eggs and J2 divided by the inoculum. The host response of each genotype to *M. incognita* was determined as follows: the genotypes with a significantly lower final nematode population density compared to the susceptible reference cultivar Grande Naine were categorised as resistant while the genotypes with a final nematode population density which was not significantly different compared to the susceptible reference cultivar Grande Naine were categorised as susceptible.

2.2.5. STATISTICAL DATA ANALYSIS

Statistical analysis was performed using STATISTICA version 7 (StatSoft, Tulsa, OK, USA). Prior to each analysis, the basic assumptions for parametric statistics, namely normal distribution and homogeneity of variances were tested (Anonymous, 2007). Based on these tests, the nematode population densities were $\log_{10}(x+1)$ transformed. Analysis of variance was performed to determine the host response of the *Musa* genotypes to *R. similis* and *M. incognita*. The final nematode population density of each genotype was compared to the final nematode population densities of the reference cultivars by the two-sided Dunnett's test ($P \leq 0.05$).

2.3. RESULTS

2.3.1. HOST RESPONSE TO *R. SIMILIS*

The results of the experiment on the host response to *R. similis* are summarised in Table 2.3. The results show that the plants had good growing conditions during the experiment and this resulted in fresh root weights ranging from an average of 22.8 g in Gia Hui to an average of 76.3 g in Pisang Jari Buaya.

Significantly ($P \leq 0.05$) higher numbers of nematodes were present in the susceptible reference cultivar Grande Naine compared to the resistant reference cultivar Yangambi km5. The final nematode population density was about 4 and 5 times higher in Grande Naine than in Yangambi km5 in the first and second batch, respectively. Lesions on Grande Naine were long, continuous and tunnel-like (Fig. 2.2A). Lesions on Yangambi km5 were short and discontinuous resembling hypersensitive lesions (Fig. 2.2B). The other resistant reference cultivar, Pisang Jari Buaya, expressed an inconsistent host response since it was resistant to *R. similis* in the first batch but its host response was inconclusive in the second batch.

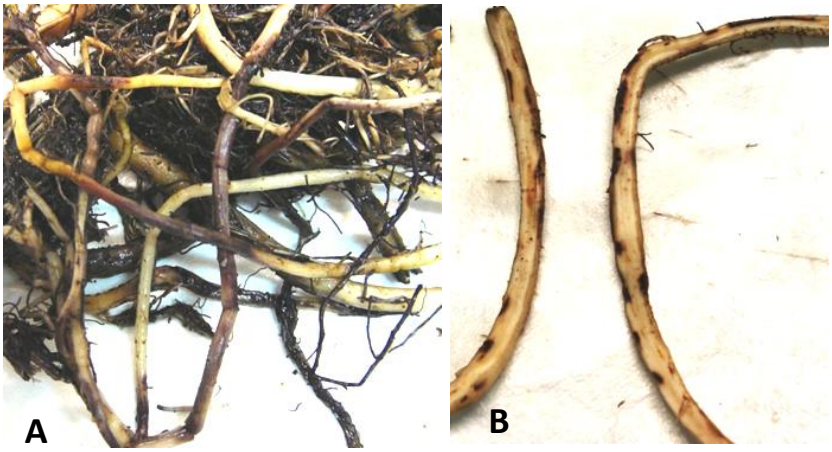


Figure 2.2: Root necrosis caused by *Radopholus similis* at 8 weeks after inoculation with 1,000 nematodes. A) Long, continuous and tunnel-like root lesions on the susceptible reference cultivar Grande Naine. B) Short, discontinuous, hypersensitive-like lesions on the resistant reference cultivar Yangambi km5.

Out of the seven *Musa* genotypes tested, Long Tavoy, Saba, Pisang Mas and Pora Pora were resistant to *R. similis* based on their final nematode population density. Marau expressed a partial resistance to *R. similis*. The host response of Kokopo was inconclusive and Gia Hui was susceptible to *R. similis*.

2.3.2. HOST RESPONSE TO *M. INCOGNITA*

The results of the experiment on the host response to *M. incognita* are summarised in Table 2.4.

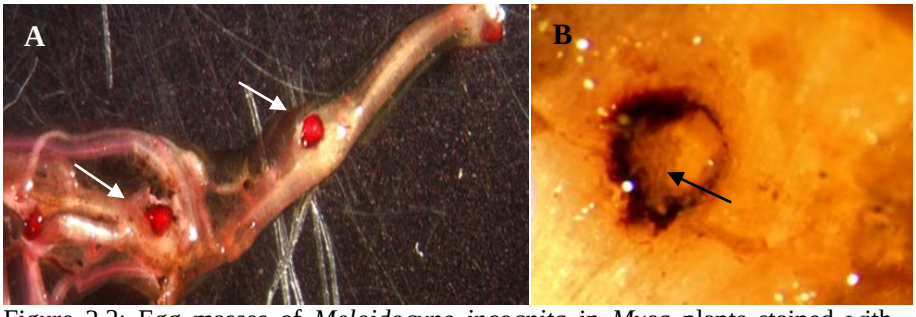


Figure 2.3: Egg masses of *Meloidogyne incognita* in *Musa* plants stained with phloxin B. A) Egg masses on a secondary root (x40). B) Egg mass inside a thick primary root surrounded by a layer of phenolic root cells (x100).

The results show that the plants had good growing conditions during the experiment and this resulted in fresh root weights ranging from an average of 11.6 g in Kokopo to an average of 45.2 g in Pisang Mas. Although characteristic galls were not very conspicuous for direct observation in our study, many egg-laying females and egg masses were observed during counting (Fig. 2.3). The susceptible reference cultivar Grande Naine had a high number of egg-laying females per 5 g roots and the highest final nematode population density per 5 g roots and per root system. Out of the eight *Musa* genotypes tested, Vudu Papau and Pisang Mas were resistant to *M. incognita* based on their final nematode population density. The number of egg-laying females per 5 g roots was low in Pisang Mas but in Vudu Papau as high as in Grande Naine. Nevertheless, the final nematode population density per 5 g roots and per root system was about 5 to 6 times lower in Vudu Papau than in Grande Naine. The other genotypes tested were susceptible to *M. incognita*.

2.4. DISCUSSION

The resistance to *R. similis* of Long Tavoy, Saba, Pisang Mas and Pora Pora observed when using sucker-derived plants (Dochez *et al*., 2006) was confirmed by our study with tissue culture-derived plants. However, in our study Gia Hui was susceptible to *R. similis* while in the study by Dochez *et al*. (2006) this genotype was resistant. Our study confirms other reports (De Waele *et al*., 1998; Stanton, 1999; Viaene *et al*., 2003; Blomme *et al*., 2001, 2003, 2004) that tissue culture-derived plants of some *Musa* genotypes could be susceptible and sensitive to *R. similis* while sucker-derived plants of the same genotype are resistant. It is possible that the *R. similis* resistance genes are not yet expressed by tissue-cultured plants at the time of infection while they are being expressed at the same time in sucker-derived plants. Previously, the host response of Pisang Mas to *R. similis* was reported as less susceptible (Binks & Gowen, 1997) and partially resistant (Marin *et al*., 2000). Conversely, another study (Stoffelen *et al*., 1999) reported Pisang Mas as susceptible to *R. similis*. These observations underline the necessity to confirm the resistant host response observed in *Musa* genotypes to *R. similis* under a range of experimental conditions, including different types of planting material (Herradura, 2009).

Table 2.3. Host response of selected *Musa* genotypes and the reference cultivars[†] to *Radopholus similis*, measured at 8 weeks after inoculation with 1,000 vermiform nematodes per plant (n = 8). Plants were inoculated at 8 weeks after planting.

Genotype	Fresh root weight (g)	Pf		Rf	Comparison of Pf/root system with		Host response
		per g roots	per root system		Grande Naine	Yangambi km5	
Batch 1							
Long Tavoy	62.2	86	5,331	5.3	*	n.s.	R
Saba	61.2	151	9,521	9.5	*	n.s.	R
Pisang Mas	53.6	277	15,079	15.1	*	n.s.	R
Marau	43.5	375	15,457	15.5	*	*	PR
Grande Naine[†]	54.7	485	26,499	26.5	-	*	S
Yangambi km5[†]	56.4	134	7,332	7.3	*	-	R
Pisang Jari Buaya[†]	76.3	150	11,350	11.4	*	n.s.	R
Batch 2							
Pora Pora	69.7	558	37,958	38.0	*	n.s.	R
Kokopo	49.9	811	38,404	38.4	n.s.	n.s.	I
Gia Hui	22.8	3,008	62,115	62.1	n.s.	*	S
Grande Naine[†]	45.2	1,543	69,444	69.4	-	*	S
Yangambi km5[†]	64.7	245	13,885	13.9	*	-	R
Pisang Jari Buaya[†]	65.7	631	40,748	40.7	n.s.	n.s.	I

Pf: final nematode population density; Rf: reproduction factor (Pf/Pi; Pi: initial nematode inoculum density).
*: significantly different; n.s.: not significantly different according to Dunnett's test ($P \leq 0.05$).
R: resistant; PR: partially resistant; S: susceptible; I: inconclusive.

Table 2.4. Host response of selected *Musa* genotypes and the susceptible reference cultivar Grande Naine to *Meloidogyne incognita* measured at 8 weeks after inoculation with 4,000 eggs and second-stage juveniles per plant (n = 8). Plants were inoculated at 8 weeks after planting.

Genotype	Fresh root weight	Egg-laying females	Final nematode population density (Pf)		Rf	Comparison of Pf per root system with Grande Naine	Host response
	(g)	per 5 g roots	per 5 g roots	per root system			
Vudu Papau	17.8	8	390	1,390	0.35	*	R
Pisang Mas	45.2	2	207	1,874	0.47	*	R
Kokopo	11.6	7	1,123	2,606	0.65	n.s.	S
Long Tavoy	34.2	4	386	2,643	0.66	n.s.	S
Saba	40.3	4	590	4,755	1.19	n.s.	S
Gia Hui	17.3	12	1,590	5,501	1.38	n.s.	S
Pora Pora	26.1	3	821	4,285	1.07	n.s.	S
Marau	18.3	9	1,634	5,979	1.49	n.s.	S
Grande Naine	22.5	8	1,990	8,954	2.24	-	-

Rf: reproduction factor (Pf/Pi; Pi: initial nematode inoculum density).
*: significantly different; n.s.: not significantly different according to Dunnett's test ($P \leq 0.05$).
R: resistant; S: susceptible.

Despite the resistance to *R. similis* shown by Long Tavoy, Saba, Pisang Mas, Pora Pora and Yangambi km5, the final nematode population densities were high in their root systems. This indicates that these resistant genotypes are not immune but significantly less susceptible to *R. similis* compared to the susceptible reference cultivar Grande Naine. In the case of complete immunity, the reproduction ratio would have been less than one as reviewed by Bos and Parlevliet (1995).

The final *R. similis* population density of Pisang Jari Buya, the other resistant reference cultivar, was significantly ($P \leq 0.05$) lower in the first batch compared to Grande Naine but not in the second batch. This observation confirms previous observations (Herradura, 2009) that the resistance to *R. similis* in Pisang Jari Buya in experiments using tissue culture-derived plants can be more easily overcome by *R. similis* compared to Yangambi km5. Pisang Jari Buaya was also reported as showing only partial resistance to *R. similis* in another study (Binks & Gowen, 1997). It indicates that Yangambi km5 is a more reliable resistant reference cultivar than Pisang Jari Buaya. However, care should be taken with this observation.

The final *M. incognita* population densities of Vudu Papau and Pisang Mas were significantly ($P \leq 0.05$) lower compared to the susceptible reference cultivar Grande Naine. Vudu Papau had on average 80.4 and 84.5%, and Pisang Mas on average of 89.6 and 79.1% less nematodes per 5 g roots and per root system, respectively, compared to Grande Naine. However, the numbers of egg-laying females on these two genotypes were not significantly different from Grande Naine. Although the mean root fresh weight of Pisang Mas was high at the end of the experiment, this did not lead to a high number of *M. incognita* per root system. This can be illustrated by the observation that the root fresh weight of Pisang Mas was 2 times higher than Grande Naine, but the final nematode population density per root system was 4.8 times lower. This observation indicates that Pisang Mas might be a good source of resistance to *M. incognita* but this need to be confirmed under field conditions. Care should be taken since the degree of resistance exhibited by these genotypes is hard to categorize due to the lack of resistant reference cultivars in *Musa*. In future experiments, Pisang Mas could be included as a resistant reference cultivar to compare *M. incognita* host responses among *Musa* genotypes. In this thesis, the mechanisms of *Radopholus similis* resistance in *Musa* genotypes are studied in the further chapters (Chapter 5 and 6). In Pisang mas, the same mechanisms could operate against the resistance of *Meloidogyne incognita* also.This can be highly interesting for future studies.

In spite of a relatively high number of studies (Stoffelen *et al*., 1999, 2000; Van den Bergh *et al*., 2006; Quénéhervé *et al*., 2008b), resistance to *M. incognita* has not been found in *Musa* genotypes except by Davide and Marasigan (1985), and Dizon *et al*. (2010). Davide and Marasigan (1985) identified nine out of 90 *Musa* genotypes evaluated as resistant to *M. incognita*. However, the host plant response of these genotypes was only based on the root gall index, not on nematode reproduction. Dizon *et al*. (2010) examined the host response to *M. incognita* of 32 Philippine banana cultivars and identified three banana cultivars as resistant to *M. incognita* based on nematode reproduction.

2.5. CONCLUSION

Our study identified Pisang Mas as a *Musa* genotype with combined resistance to both *R. similis and M. incognita*. This is the first time that a *Musa* genotype has been found as resistant to these two major banana root pathogens. Resistance of Long Tavoy, Saba, Pisang Mas and Pora Pora to *R. similis* was confirmed. Three *R. similis* resistant *Musa* genotypes, Long Tavoy, Saba, Yangambi km5 were shortlisted for characterization of *R. similis* resistance in comparison with the susceptible reference cultivar Grande Naine. These three genotypes were selected based on the low final *R. similis* population density in their roots.

CHAPTER 3

DEVELOPMENT OF AN AUTOTROPHIC *IN VITRO* MODEL SYSTEM TO STUDY *RADOPHOLUS SIMILIS* HOST LOCATION AND PENETRATION

3.1. INTRODUCTION

Some nematode management strategies such as the use of resistant host plants aim at the disruption of the life and infection cycles of plant-parasitic nematodes. Understanding how and when resistant plants interfere with the attraction and migration of plant-parasitic nematodes towards roots, and with nematode penetration, development and reproduction inside the roots may lead to practices that increase the efficacy of the nematode management strategy or the development of novel nematode management strategies (Sheridan *et al*., 2004).

All plant-parasitic nematodes attacking roots are soil-borne. Studying the nematode's behaviour in soil has been very difficult because it is in most cases impossible to view the nematodes *in situ* because of their microscopic size, transparent body and the opaque soil habitat (Spence *et al*., 2008). To study the behaviour of plant-parasitic nematodes in the soil, model systems are necessary.

In autotrophic *in vitro* systems, plant shoots are exposed to open air allowing the plant to perform active photosynthesis through atmospheric carbon exchange. At the same time, the root system and the nematodes are maintained under strict *in vitro* conditions in a Petri dish. Due to the horizontal root growth in the Petri dishes, this system enables direct observation of the roots and the nematodes using a stereoscopic microscope. A successful autotrophic *in vitro* system was developed by Van der Veken (2010) to study the bio-protective effect of *Rhizobium etli* against *Meloidogyne incognita* on common bean (*Phaseolus vulgaris*). In a strict *in vitro* system, the whole plant is grown inside a culture tube hence the plant cannot perform active photosynthesis by atmospheric carbon exchange. The culture medium supplies carbon source to these plants in the form of sucrose. The results obtained from this model system are applicable to the field conditions as the banana plants in the field are composed of young and old roots. Banana plants produce fresh roots continuously until flowering (Swennen *et al*., 1986) and especially nematodes prefer to enter the root system through the young roots and the root tips (Sarah *et al*., 1996).

In this part of our study we report the development of an autotrophic *in vitro* model system to study the host location and penetration behaviour of *Radopholus similis* (Cobb, 1893) Thorne, 1949 on bananas. The basic set-up of our system was based on the autotrophic *in vitro* system developed by Koffi *et al*. (2009) to study banana mycorrhization.

3.2. MATERIALS AND METHODS

3.2.1. PLANTING MATERIAL

Tissue-cultured banana (*Musa acuminata*) plants of the cultivars Grande Naine (*Musa acuminata* AAA, Cavendish subgroup, ITC 1256), Yangambi km5 (*Musa acuminata* AAA, Ibota subgroup, ITC 1123) and Saba (*Musa* spp. ABB, Saba subgroup, ITC 1138), were initially obtained from the *Musa* germplasm collection maintained at the International Transit Centre (ITC), K.U.Leuven, Belgium. The plant material was proliferated, regenerated and rooted as explained in Chapter 2 (2.2.2.). Shoots of well-developed plantlets were transferred to sterile culture boxes (8 x 5.5 x 10 cm) containing 150 ml of Murashige and Skoog medium including vitamins, 10 mg/l ascorbic acid, 30 g/l sugar and 2 g/l gelrite with pH 6.12 to allow horizontal root growth (Fig. 3.1A). The plantlets in the sterile boxes were grown in growth chambers at 28 °C and 16 h photoperiod. After 3 weeks, plantlets with developing secondary roots were transferred to the autotrophic *in vitro* system.

3.2.2. NEMATODE INOCULUM

A population of *R. similis* originally isolated from banana roots in Uganda was used in the experiments. The population from Uganda was characterized by a high reproductive fitness (Fallas *et al*., 1995). This population was maintained and multiplied monoxenically on sterile alfalfa callus tissues at 25±1 °C in the dark (Elsen *et al*., 2001). To obtain inoculum, nematodes were collected from the callus tissues or from the medium by rinsing with sterile distilled water. Only 6-weeks-old nematode cultures were used to prepare the inoculum.

3.2.3. THE AUTOTROPHIC *IN VITRO* SYSTEM

For the autotrophic *in vitro* system, sterile Petri dishes with a diameter of 14.5 cm were used. The dishes were filled with 150 ml of Murashige and Skoog medium including vitamins, 10 mg/l ascorbic acid and 2 g/l gelrite with pH 6.12 but lacking sugar. A 1-cm-diameter perforation was made on the edge of the Petri dish lid using a heated cork borer. Well-rooted, 3-weeks-old plantlets from the culture boxes (Fig. 3.1A) were transferred carefully to the Murashige and Skoog medium at an edge of the Petri dishes. Placing the plants in the edge did not affect the growth of the plants as previously tested with mulching the banana plants in either side of the root system (Swennen, 1984).

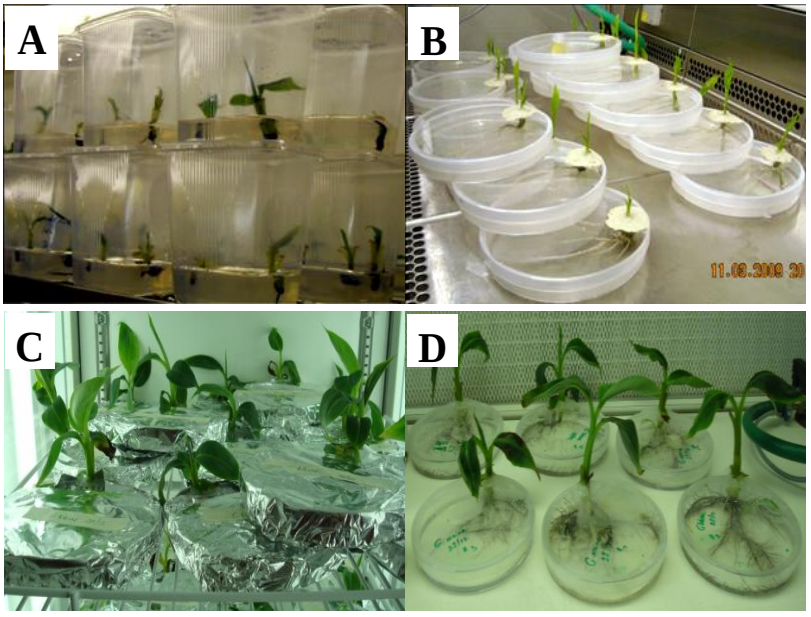


Figure 3.1: Banana plants cultured in an autotrophic *in vitro* model system. A) Pre-rooting of the plantlets in culture boxes for 3 weeks. B) Pre-rooted plantlets transferred to the autotrophic *in vitro* system. C) Plants in the phytotron incubator. D) Plants cultured in the autotrophic *in vitro* system for 4 weeks.

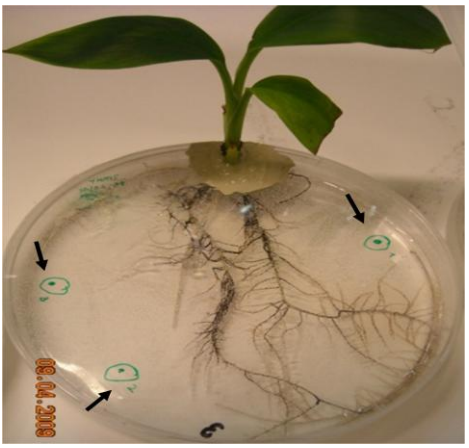


Figure 3.2: *Radopholus similis* inoculation spots (arrows) to study nematode attraction and penetration in an autotrophic *in vitro* model system.

Each pre-rooted plantlet was positioned vertically in the Petri dish, with the roots on the surface of the Murashige and Skoog medium and the shoot in upright position. The Petri dishes were then covered with the perforated lids allowing the shoots to extend out in the air through the perforations (Fig. 3.1B).

The perforations were plastered with sterile sealer BREATHseal™ (Greiner Bio-one) and additionally sealed with steam-sterilized silica gel. All handlings were conducted in a laminar air flow chamber. The Petri dishes were sealed with parafilm, covered with aluminium foil and placed in a phytotron incubator (Fig. 3.1C). The ambient conditions in the incubator were 75% relative humidity, 27/25 °C (day/night) and 12 h photoperiod. After 4 weeks, when the plants had developed a good root system with secondary and tertiary roots (Fig. 3.1D), they were ready for use in the experiments.

3.2.4. EXPERIMENTAL SET-UP

Four experiments were carried out. The first two experiments were optimisation experiments to optimise the system. In these experiments, the attraction and penetration of *R. similis* towards and in single plants of two resistant *Musa* genotypes, Yangambi km5 and Saba, were examined. When the system was optimised, two more experiments were performed. In the third experiment, the attraction and penetration of *R. similis* towards and in single plants of Grande Naine and Yangambi km5, a susceptible and resistant *Musa* genotype, respectively, were examined. In the fourth experiment, a newly developed two-compartment system was used to examine the attraction of *R. similis* to either Grande Naine or Yangambi km5 when both *Musa* genotypes were present. All experiments were conducted with 6 replications and randomized in a completely randomized design (CRD). In the optimisation experiment, the autotrophic *in vitro* plants were grown in growth chambers at 28 °C and 16 h photoperiod. But few tested plants showed that the autotrophic in vitro Plant grew very well in a phytotron incubator compared to the plants grown in growth chambers. Hence, for the third and fourth experiments, the autotrophic *in vitro* plants were grown in a phytotron incubator with the ambient conditions mentioned in section 3.2.3.

A) Optimisation experiments with resistant plants
   nematode attraction towards individual plants
   nematode penetration in individual plants
B) Comparing resistant and susceptible plants
   nematode attraction towards and penetration in individual plants
   nematode attraction in a two-compartment system

3.2.4.1. First experiment: attraction

In this first (optimisation) experiment, six well-rooted (roots that developed secondary and few tertiary roots) four-weeks-old autotrophic *in vitro* plants each of Yangambi km5 and Saba were inoculated with 30 mature (gravid) *R. similis* females. Nematodes were collected one by one using a sterile glass micropipette and placed in a small drop of sterile water on the medium near the plants. The nematode inoculum was equally distributed over three inoculation spots per plate. The inoculation spots were situated at 2 cm from the roots (Fig. 3.2). The lids of the Petri dishes were slightly opened for nematode inoculation. All handlings were conducted in a laminar air flow chamber. At 1, 2, 4 and 24 h after inoculation, the number of nematodes that had migrated towards the roots was counted using a stereoscopic microscope in a laminar air flow chamber and expressed as a percentage of the inoculated nematodes.

3.2.4.2. Second experiment: penetration

In this second (optimisation) experiment, two sets of six well-rooted autotrophic *in vitro* plants of each Yangambi km5 and Saba were inoculated with 30 mature (gravid) *R. similis* females close to the roots as described above.

At 2 and 4 days after inoculation, the roots were carefully removed from the medium, washed, blot-dried and weighed. Then, the roots were cut into 1-cm-pieces, stained with acid fuchsin and mounted on glass slides (Hooper *et al.*, 2005). The nematodes that had penetrated the roots were counted using a light microscope[3] and expressed as a percentage of the inoculated nematodes.

3.2.4.3. Third experiment: attraction and penetration

In the third experiment, two sets of six well-rooted autotrophic *in vitro* plants of each Grande Naine and Yangamibi km5 were inoculated with 30 mature (gravid) *R. similis* females as described above. At 1, 3, 4 and 6 h after inoculation, the number of nematodes that had migrated towards the roots was counted using a stereoscopic microscope in a laminar air flow chamber and expressed as a percentage of the inoculated nematodes. The plants were placed in the phytotron incubator after counting for nematode attraction.

At 2 and 4 days after inoculation, the roots were carefully removed from the medium, washed, blot-dried and weighed. Then, the roots were cut into 1-

[3] All microphotographs in this chapter were taken using an EVOS® XL microscope camera and micron (EVOS) imaging software (Advanced Microscopy Group, Bothell, WA, USA).

cm-pieces, stained with acid fuchsin and mounted on glass slides (Hooper *et al.*, 2005). The nematodes that had penetrated the roots were counted using a light microscope and expressed as a percentage of the inoculated nematodes.

3.2.4.4. Fourth experiment: attraction and penetration in a two-compartment system

In the fourth experiment, six well-rooted autotrophic *in vitro* plants each of Grande Naine and Yangambi km5 were connected with a small transparent bridge at the start of the experiment (Fig. 3.3).

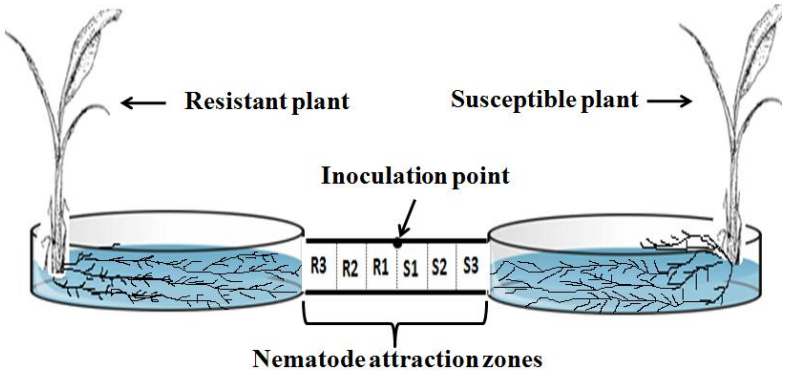


Figure 3.3: Two-compartment autotrophic *in vitro* model system to study nematode attraction to banana plants. R1, R2 and R3 represent the attraction zones of the resistant plant. S1, S2 and S3 represent the attraction zones of the susceptible plant.

Figure 3.4: Two-compartment autotrophic *in vitro* model system to study nematode attraction to banana plants.

The bridge was 7 cm long, 1 cm wide and contained approximately 4.5 ml of 1% sterile water agar. The middle of the bridge was selected as the inoculation spot. Three attraction zones were marked off at each side of the bridge based on their distance from the inoculation spot (Table 3.1). The zones

at the side of Yangambi km5, the resistant genotype, are denoted as R1, R2 and R3 while the zones at the side of Grande Naine, the susceptible genotype, are denoted as S1, S2 and S3 (Fig. 3.3). To connect the Petri dishes to the bridge, 1-cm-diameter openings were made on the edge of the Petri dishes using a heated forceps. The bridges were secured firmly using transparent sticky tapes (Fig. 3.4).

Table 3.1. Nematode attraction zones in the two-compartment autotrophic *in vitro* model system.

Zone	Distance from the inoculation spot
1	0-1.0 cm
2	1-2.0 cm
3	2-3.5 cm

At 3 h after the two compartments were connected *via* the bridge, 30 mature (gravid) *R. similis* females were inoculated in a small depression made at the inoculation spot. At 30 min, 1, 2, 3 and 6 h after inoculation, the number of nematodes that had migrated to each attraction zone was counted using a stereoscopic microscope in a laminar air flow chamber and expressed as a percentage of the inoculated nematodes.

3.2.5. STATISTICAL DATA ANALYSIS

Data with normal distribution and homogeneous variances were subjected to analysis of variances using STATISTICA version 9 (StatSoft, Tulsa, OK, USA). Percentages (percentage nematode attraction and penetration) were arcsin (x/100) transformed prior to the analysis. When significant differences ($P \leq 0.05$) were observed, Tukey's HSD (Honestly Significant Difference) test was applied for multiple comparisons of group means.

3.3. RESULTS

3.3.1. FIRST (OPTIMISATION) EXPERIMENT: ATTRACTION

Migration of the nematodes towards the roots was observed (Fig. 3.5). Plants had good growth in the absence of sugar due to active photosynthesis. At 1 h after inoculation, a significantly ($P \leq 0.05$) higher percentage of *R. similis* had migrated towards the roots of Saba compared to Yangambi km5 (52.6 *vs* 6.7%)

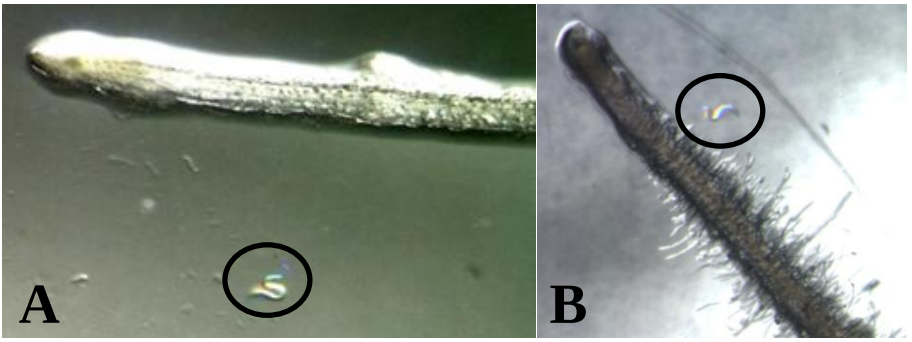


Fig. 3.5: Migration of *Radopholus similis* females towards the roots of A) Yangambi km5 and B) Saba at 1 h after inoculation. Magnification: 40x

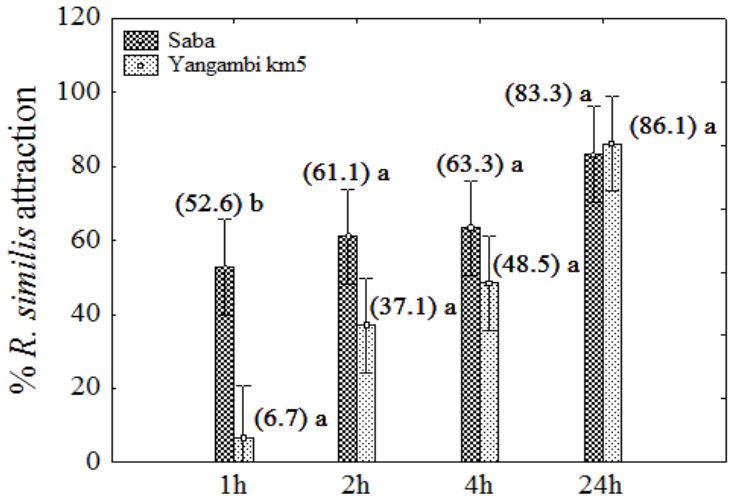


Figure 3.6: Attraction of *Radopholus similis* females (expressed as a percentage of 30 inoculated mature females) towards the roots of 4-weeks-old plants of the *Musa* genotypes Yangambi km5 and Saba at 1, 2, 4 and 24 h after inoculation. Means are presented between parentheses. Means within a same time (h) followed by the same letter are not significantly ($P \leq 0.05$) different from each other according to Tukey's HSD test. Error bars represent confidence intervals. (n=6).

At 2, 4 and 24 h after inoculation, no significant differences in migration towards the roots of both *Musa* genotypes were observed anymore. The difference between the two genotypes decreased at each observation time and at 24 h after inoculation, more than 80% of the inoculated nematodes had migrated towards the roots of Saba as well as Yangambi km5 (Fig. 3.6).

3.3.2. SECOND (OPTIMISATION EXPERIMENT): PENETRATION

Plant growth in the autotrophic *in vitro* system was good (Table 3.2). A small decrease in fresh root and shoot weights was observed at 4 days after inoculation (DAI) compared to 2 DAI but the differences were not statistically significant. No significant differences were observed between the percentages of nematodes that had penetrated the roots of Saba and Yangambi km5 at 2 and 4 DAI. At 2 DAI, 30 and 25.7% of the 30 inoculated females had penetrated the roots of Saba and Yangambi km5, respectively (Fig. 3.7).

Table 3.2. Fresh root and shoot weights of 4-weeks-old plants of the *Musa* genotypes Saba and Yangambi km5 at 2 and 4 days after inoculation (DAI) with 30 mature females of *Radopholus similis*.

Genotypes	Fresh root weight (g)		Fresh shoot weight (g)	
	2 DAI	4DAI	2 DAI	4DAI
Saba	0.88	0.64	1.26	1.18
Yangambi km5	0.58	0.44	1.60	1.38

Means within the same columns followed by the same letter are not significantly ($P \leq 0.05$) different from each other according to Tukey's HSD Test. Data were $\log_{10}(x+1)$ transformed prior to statistical analysis.

Although more than 80% of the females had been attracted and migrated towards the roots (Fig 3.6), only about 25 to 30% of the females had penetrated the roots at 2 DAI (Fig. 3.7A). Penetration of a single root by many females at the same site was observed (Fig. 3.8B). Penetrated adult females were laying eggs at 4 DAI in both Saba and Yangambi km5 (Fig. 3.8). At 4 DAI, no significant differences were observed in the number of eggs laid in Saba and Yangambi km5 (Fig. 3.7B). As no increase in penetration was observed at 4 DAI compared with 2 DAI, the observation time to examine nematode penetration was limited to 2 DAI in the next experiment.

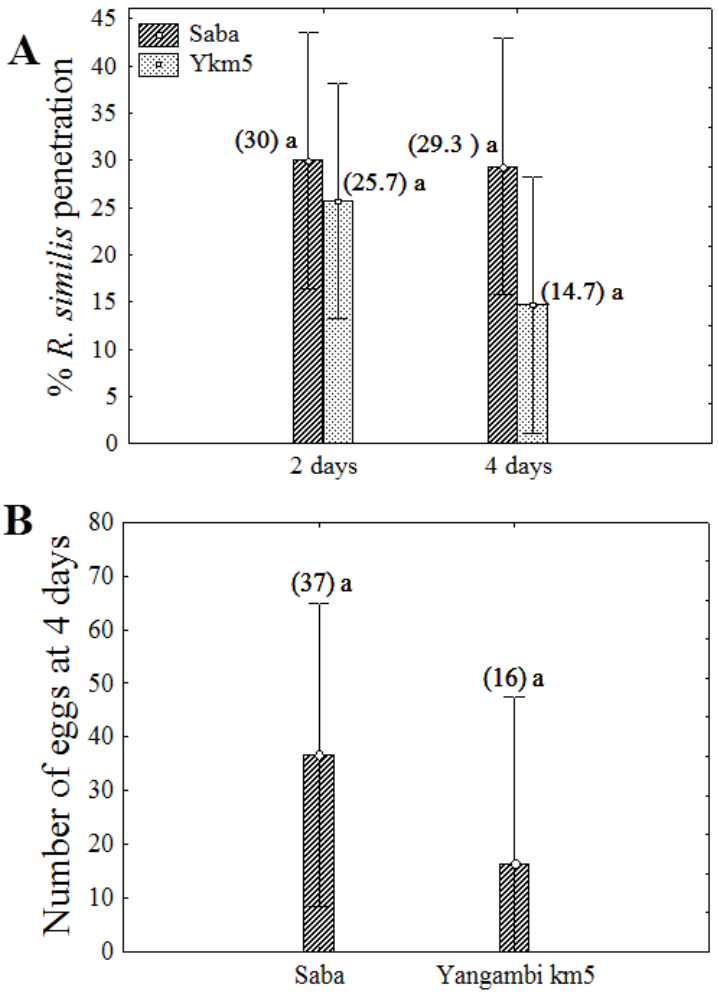


Figure 3.7: A) Penetration of *Radopholus similis* females (expressed as a percentage of 30 inoculated mature females) in the roots of 4-weeks-old plants of the *Musa* genotypes Yangambi km5 and Saba at 2 and 4 days after inoculation. B) Number of eggs laid by the penetrated *R. similis* females at 4 days after inoculation. Means are presented between parentheses. Means within a same time (days) followed by the same letter are not significantly ($P \leq 0.05$) different from each other according to Tukey's HSD test. Error bars represent confidence intervals. (n=6).

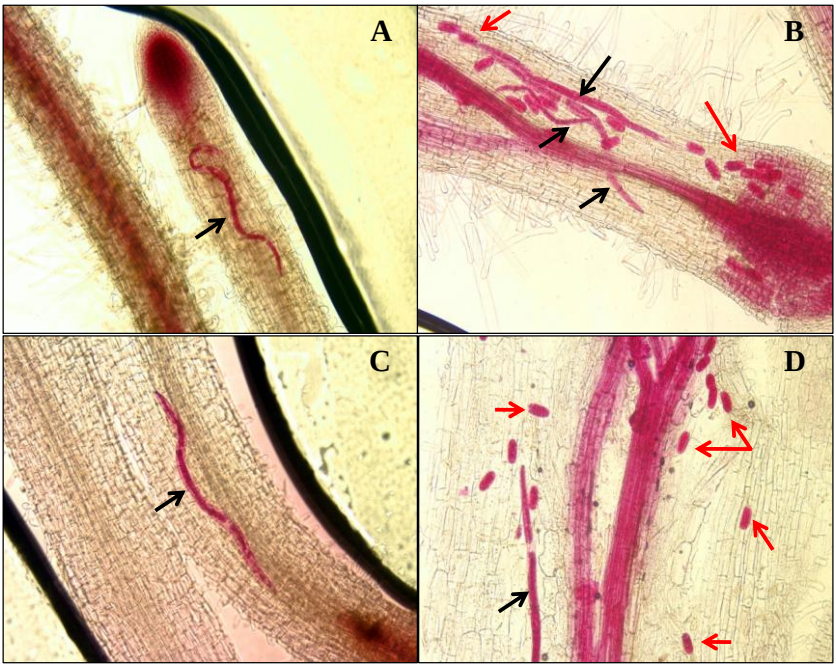


Figure 3.8: Penetration and egg laying of *Radopholus similis* females in banana roots grown in an autotrophic *in vitro* system. Roots of Saba at A) 2 days and B) 4 days after inoculation with 30 females and roots of Yangambi km5 at C) 2 days and D) 4 days after inoculation with 30 females. Black arrows indicate females and red arrows indicate eggs. Females and eggs were stained with acid fuchsin. Magnification: 40x.

3.3.3. THIRD EXPERIMENT: ATTRACTION AND PENETRATION

At 1 h after inoculation, a significantly ($P \leq 0.05$) higher percentage of females had migrated towards the roots of the susceptible genotype Grande Naine compared to the resistant genotype Yangambi km5 (71.5 *vs* 25.4%, respectively). At 3, 4 and 6 h after inoculation, no significant differences in migration towards the roots of both *Musa* genotypes were observed anymore. The difference between the two genotypes decreased at each observation time and at 4 h after inoculation, more than 80% of the inoculated nematodes had migrated towards the roots of Grande Naine as well as Yangambi km5 (Fig. 3.9).

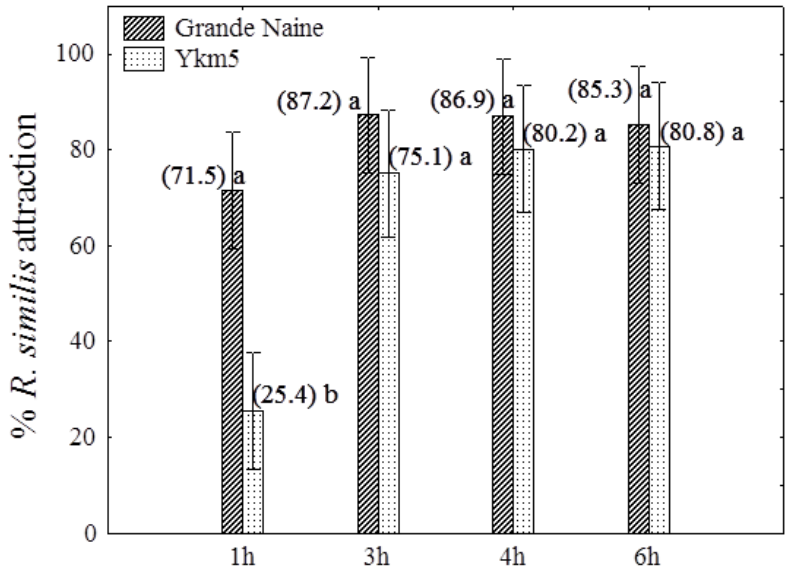


Figure 3.9: Attraction of *Radopholus similis* females (expressed as a percentage of 30 inoculated mature females) towards the roots of 4-weeks-old plants of the susceptible *Musa* genotype Grande Naine and the resistant *Musa* genotype Yangambi km5 at 1, 3, 4 and 6 h after inoculation. Means are presented between parentheses. Means within a same time (h) followed by the same letter are not significantly ($P \leq 0.05$) different from each other according to Tukey's HSD test. Error bars represent confidence intervals. (n=6).

Plant growth in the autotrophic *in vitro* system was good (Table 3.3). The percentages of females that had penetrated the roots of Yangambi km5 and Grande Naine were not significantly different from each other at both 1 and 2 DAI (Fig. 3.10).

Table 3.3. Fresh root and shoot weights of 4-weeks-old plants of the *Musa* genotypes Grande Naine and Yangambi km5 at 1 and 2 days after inoculation (DAI) with 30 mature females of *Radopholus similis*.

Genotype	Fresh root weight (g)		Fresh shoot weight (g)	
	1 DAI	2 DAI	1 DAI	2 DAI
Grande Naine	5.0	4.8	5.2	4.6
Yangambi km5	2.9	3.3	3.1	3.8

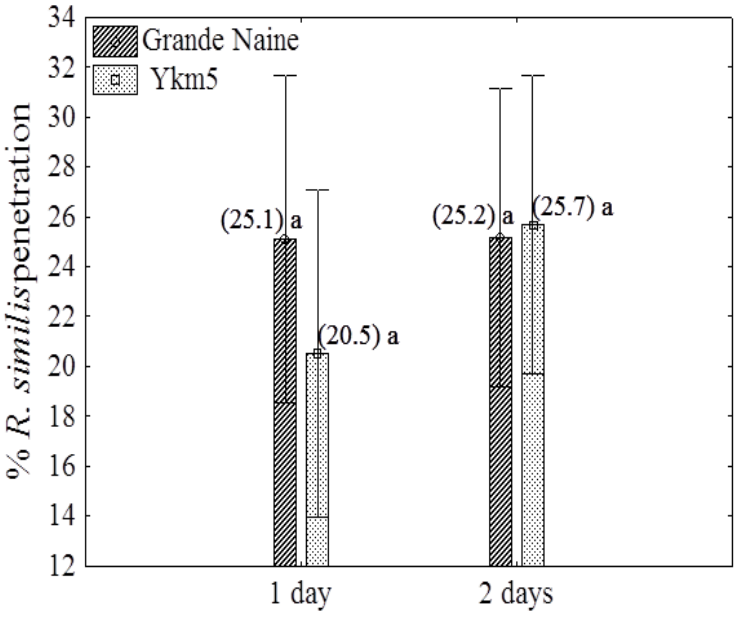


Figure 3.10: Penetration of *Radopholus similis* females (expressed as a percentage of 30 inoculated mature females) in the roots of 4-weeks-old plants of the susceptible *Musa* genotype Grande Naine and the resistant *Musa* genotype Yangambi km5 at 1 and 2 days after inoculation. Means are presented between parentheses. Means within a same time (days) followed by the same letter are not significantly ($P \leq 0.05$) different from each other according to Tukey's HSD test. Error bars represent confidence intervals. (n=6).

Although more than 80% of the females had been attracted and migrated towards the roots (Fig 3.9), only about 25% of the females had penetrated the roots at 2 DAI (Fig. 3.10). The nematode migration tracks observed in the penetration experiment indicate that the nematodes quickly sensed and migrated towards the host roots. However, at close proximity of roots, they were circling around or near the roots probably with repeated efforts to exactly locate and penetrate the root cells (Fig. 3.11).

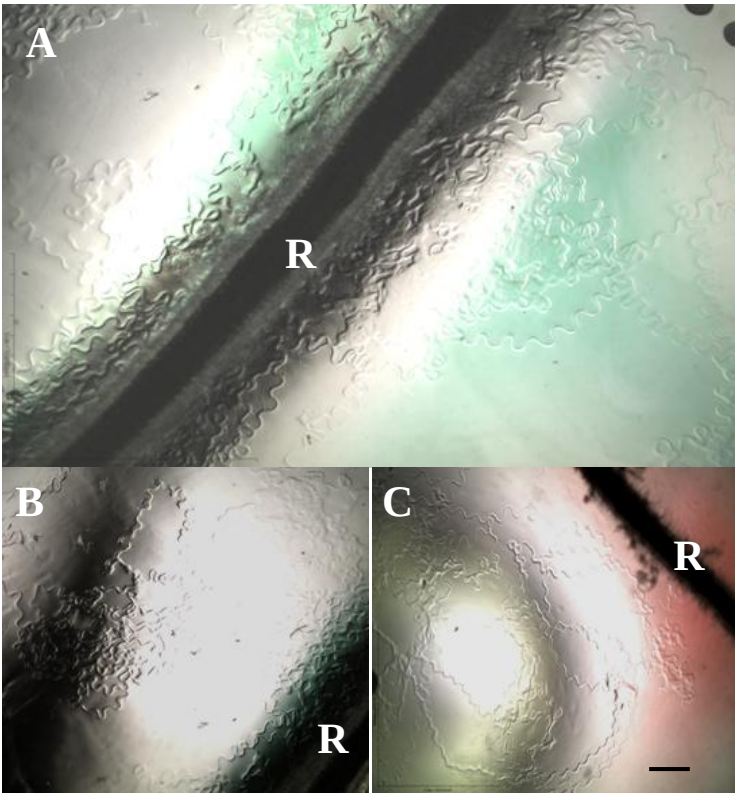


Figure 3.11: Migration tracks of *Radopholus similis* females near banana roots in an autotrophic *in vitro* system observed at 24 h after inoculation. A) and B) Grande Naine. C) Yangambi km5. At the beginning of the experiment, 30 mature females were placed at 2 cm distance from the roots. R: roots. Scale bar: 200µm

3.3.4. FOURTH EXPERIMENT: NEMATODE ATTRACTION IN A TWO-COMPARTMENT SYSTEM

The results of this experiment are shown in Fig. 3.12. At 30 min and 1 h after inoculation, the majority of the inoculated *R. similis* females had remained at the inoculation point except a small percentage (about 2%) of the nematodes that had migrated to the first attraction zones (R1 & S1).

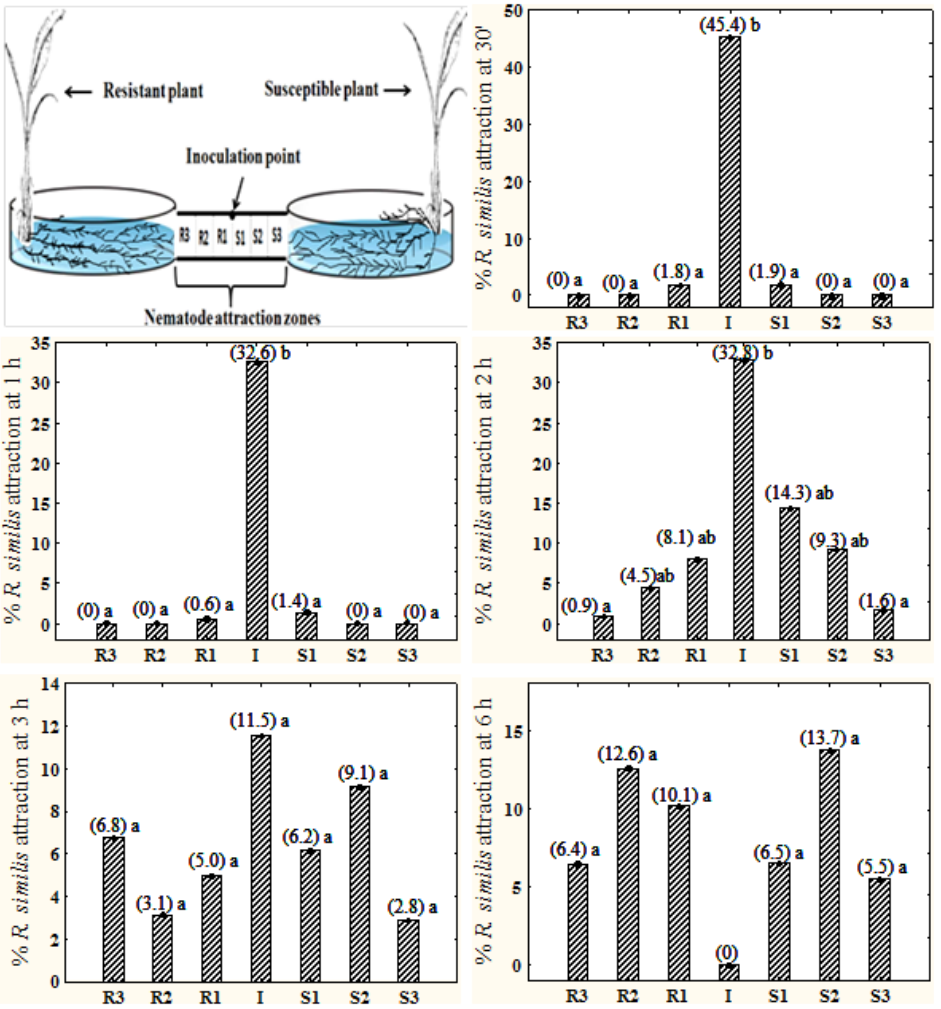


Figure 3.12: Percentages of *Radopholus similis* females present in the attraction zones of the two-compartment autotrophic *in vitro* system at 30 min, 1, 2, 3 and 6 h after inoculation of 30 mature females at the inoculation spot (I). R1, R2 and R3 represent the attraction zones of the resistant *Musa* genotype Yangambi km5 while S1, S2 and S3 represent the attraction zones of the susceptible *Musa* genotype Grande Naine. Means are presented between parentheses. Means followed by the same letters are not significantly ($P \leq 0.05$) different from each other according to Tukey's HSD test. (n=6).

No significant differences were observed between the numbers of nematodes that had migrated either to R1 or S1. At 2 h after inoculation, the first nematodes had migrated to the zones of attraction nearest to the Petri dishes (R3 and S3). At 6 h after inoculation, no nematodes were observed in the inoculation spot anymore as all the nematodes had migrated to the attraction

zones. No significant differences were observed between the numbers of nematodes that had migrated towards the different attraction zones at 3 and 6 h. About 30% of the females had been attracted to the zones of the resistant genotype Yangambi km5 while about 25% of the females had been attracted to the zones of the susceptible Grande Naine.

3.4. DISCUSSION

The autotrophic *in vitro* system proved to be a very good model system with notable advantages over the strict *in vitro* model systems to study nematode attraction towards and penetration in the roots of banana. First, in case of the strict *in vitro* systems, banana plants are usually grown inside glass tubes or jars. As a result their roots are not suitable for non-destructive microscopic observation. In an autotrophic *in vitro* system, the plant roots develop in a thin gel layer in a Petri dish. This enables direct observation of the nematodes using a stereoscopic microscope while the plant is developing intact and undisturbed. Second, the sucrose-free medium and the exposure of the shoots to open air allowed the plants to perform active photosynthesis through atmospheric carbon exchange. Thus, this system simulates more of the plant's natural conditions when compared to the strict *in vitro* systems. Third, the autotrophic *in vitro* system does not compromise the advantages of the strict *in vitro* systems such as providing controlled experimental conditions, limited space requirements, reduced experimental duration and easy handling (Elsen *et al.*, 2002). Hence, large batches of plants can be studied in a short time. This system could also be used to screen banana plants for nematode resistance. Banana plants were grown successfully for about 11 weeks in this system (Koffi *et al*., 2009).

However, the autotrophic *in vitro* system demands continuous monitoring as the vigorous growth of the shoots disturbs the silica gel which is sealing the perforation on the lid. This makes the system sensitive to microbial contamination. Contaminations were prevented by re-applying the sterile silica gel whenever there is a possibility of exposure. As the vigorous root growth also depletes the medium, it is necessary to refill the medium especially in long duration experiments such as screening for nematode resistance. Careful extrapolation of the results to field situation should be done as nematode movement in the two-dimentional agar based medium is unlike the three-dimentional natural soil environments (Spence *et al.*, 2008).

Results from our study show that the *R. similis* females were attracted to both the resistant and susceptible *Musa* genotypes included in our experiments

within the first hour after infection. The low percentage of nematode attraction observed towards Yangambi km5 roots at 1 h after inoculation might be due to reduced secondary and tertiary root growth compared to Saba and Grande Naine. Nematode attraction is halted when the root's growth is stopped or limited (Perry, 1996). In general, no differences were observed in host location of resistant and susceptible plants by *R. similis* females when tested separately. Furthermore, in the two-compartment system, *R. similis* females showed no preference to migrate towards the roots of either the resistant or susceptible *Musa* genotype when a choice was given.

Concentrated, circling nematode migration tracks were observed at close proximity of the banana roots in our study. This may be because of a high concentration of root exudates or attraction cues near the roots making it difficult for the nematodes to exactly locate the roots. The nematodes seemed to make repeated attempts to penetrate the roots.

A general drop in the percentage nematode penetration was observed after initial penetration. Though insignificant, this trend is also observed in the greenhouse experiments reported in the following chapter (4.3.1 & Fig. 4.3). Emigration of certain nematodes could be a probable reason. Drastic drop in nematode penetration after initial penetration of tobacco roots by *M. incognita* was reported previously. It was attributed to nematodes emigrated after unsuccessful attempts to establish feeding sites (Schneider, 1991). Windham & Williams (1994) reported probable emigration of J2 from resistant and susceptible corn genotypes. Emigration of *M. arenaria* juveniles were reported from resistant and susceptible myrobalan plum cultivars (Voisin *et al*., 1999).

Penetration of same root by several nematodes was observed in our study. Secretions from nematode-damaged plant cells or pheromones from the nematode were reported as possible reasons (Wang *et al*., 2009)

No differences were observed in the penetration of the resistant and susceptible *Musa* genotypes included in our experiments by *R. similis* females. Despite the fact that most of the inoculated females migrated to the plant roots within a few hours after inoculation, a relatively small fraction penetrated the roots successfully. Data on the attraction and penetration of *R. similis* are scarce. Studies on root-knot nematodes (*Meloidogyne* spp.) report a similar ratio of nematode penetration in other crops. For instance, less than 10% of the inoculated *M. incognita* were reported to penetrate susceptible as well as resistant genotypes of cotton (Faske & Starr, 2009), *M. arenaria, M. hapla, M. incognita* and *M. javanica* in cucumber (Walters *et al*., 2006), and *M. arenaria* in soybean (Pedrosa *et al*., 1996). Fogain (2000b) reported no difference in the penetration rates of *R. similis* on Yangambi km5 and French Sombre, a

susceptible plantain. Similarly, no differences were reported in the penetration rates of *R. similis* in susceptible and resistant *Musa* cultivars based on an *in vitro* study but using excised roots, not intact plants (Dochez, 2004). Conversely, a higher rate of *R. similis* penetration was reported in the roots of the susceptible *Musa* genotype Poyo compared to Yangambi km5 at 24 h after inoculation (Valette *et al*., 1997). It is generally accepted that plant-parasitic nematodes are attracted by the host roots and that the sensitivity of the nematodes for this is very high. Generally, the attraction does not appear to be selective (Prot, 1980; Perry, 1996).

3.5. CONCLUSIONS

The autotrophic *in vitro* model system was a very good system with notable advantages over the strict *in vitro* systems to study nematode attraction and penetration. No differences were observed in host location of the resistant and susceptible *Musa* genotypes included in our experiments by *R. similis* females except at 1h after inoculation. Furthermore, *R. similis* females showed no preference to migrate towards the roots of either the resistant or susceptible *Musa* genotypes when a choice was given to migrate towards both plant roots at the same time. Also no differences were observed in the penetration of the resistant and susceptible *Musa* genotypes included in our experiments by *R. similis* females.

Greenhouse experiments should confirm the results observed *in vitro*. Therefore a greenhouse study was conducted and the results of this study on nematode penetration, development and reproduction of *R. similis* on resistant and susceptible *Musa* genotypes are presented in Chapter 4.

CHAPTER 4

HOST PLANT RESISTANCE IN *MUSA* GERMPLASM TO *RADOPHOLUS SIMILIS*: PRE- OR POST-INFECTIONAL?

4.1. INTRODUCTION

The successful infection of susceptible *Musa* genotypes by *Radopholus similis* (Cobb, 1893) Thorne, 1949 involves many compatible nematode-plant interactions eventually leading to damage and yield loss. Although these interactions are dynamic and continuous, specific phases can be distinguished. The first phase is the location of the host plant and migration towards the roots of the host plant which are based, among many factors, on cues released by the host plant such as root exudates (Perry, 1996; Spence *et al*., 2008). The root and root cap exudates are rich in ions, enzymes, mucilage, secondary metabolites such as IAA, volatile compounds as well as peptides and plant primary metabolites (Curtis 2008; Wang *et al*., 2011). Next, the nematodes penetrate the root cell walls with the aid of a stylet and cell wall degrading enzymes (Haegeman *et al*., 2008, 2009). Then, after entering the roots, the nematodes feed continuously and develop into further life stages, and finally reproduce.

In contrast, resistant plants interfere in the nematodes' life and infection cycles ultimately resulting in an incompatible plant-nematode interaction. This process also involves many host responses limiting the nematodes (reviewed by Fuller *et al*., 2008). Host plant resistance to nematodes may occur either at the pre- or post-infectional level (Kaplan & Keen, 1980).

When the resistance operates at the pre-infectional level, the nematodes' attraction and/or migration towards the roots of the host plant or penetration into the roots of the host plant is hampered. The host plant can release cues such as metabolites or peptides that block the nematodes' sensory perception disrupting the orientation of the nematodes towards the roots (Wang *et al*., 2011). The host plant can resist penetration of the root cell walls by constitutive or induced production or deployment of structural defence barriers such as lignin and suberin (Valette *et al*., 1998).

In association with or apart from these early host plant responses, a stronger defence response can occur at the post-infectional stage when the nematodes have breached the first line of physical and chemical defences. Post-infectional resistance mechanisms are usually characterized by impaired nematode development and reproduction caused by various mechanisms (Zacheo *et al*., 1997; Fuller *et al*., 2008). These resistance mechanisms include the increased production of anti-nematode products or toxins such as phytoalexins, oxidised phenolics, lipid peroxides, hydrolytic enzymes and proteinases-inhibitors in the feeding cells, and hypersensitive responses.

The objective of this part of our study was to enhance our basic understanding of the resistance mechanism(s) in *Musa* spp. to *R. similis*. To achieve this objective, penetration, development and reproduction of *R. similis* in three resistant *Musa* genotypes, Long Tavoy (*Musa acuminate* AA, Burmannica subgroup, ITC 0283), Saba (*Musa* spp. ABB, Saba subgroup, ITC 1138) and Yangambi km5 (*Musa acuminata* AAA, Ibota subgroup, ITC 1123), and one susceptible *Musa* genotype, Grande Naine (*Musa acuminata* AAA, Cavendish subgroup, ITC 1256), were examined and compared. The resistant *Musa* genotypes were selected based on the results reported in Chapter 2. They showed good growth under the experimental conditions and expressed a strong resistance to *R. similis*.

4.2. MATERIALS AND METHODS

4.2.1. EXPERIMENTAL SET-UP

Two independent greenhouse experiments were conducted. In the first experiment, the attraction and penetration of *R. similis* towards and in the roots of three *R. similis*-resistant *Musa* genotypes (Long Tavoy, Saba, Yangambi km5) and one susceptible *Musa* genotype (Grande Naine) were examined. In the second experiment, the development and reproduction of *R. similis* was examined in the same four *Musa* genotypes. In the greenhouse, the ambient day and night temperatures were 27 and 20 ºC, respectively, the relative humidity was 80% and the photoperiod was 12 h.

4.2.2. PLANTING MATERIAL

All *Musa* genotypes were initially obtained from the *Musa* germplasm collection maintained at the International Transit Centre (ITC), K.U.Leuven, Belgium. The plant material was proliferated and rooted in test tubes as explained in Chapter 2 (2.2.2). The plantlets were grown in growth chambers at 28 °C and 16 h photoperiod.

4.2.3. NEMATODE INOCULUM

A population of *R. similis* originally isolated from banana roots in Uganda was used in the experiments. This population was maintained and multiplied monoxenically on sterile carrot discs at 25±1 °C in the dark (Speijer & De Waele, 1997). The population from Uganda was characterized by a high reproductive fitness (Fallas *et al*., 1995). To obtain the inoculum, *R. similis* was

extracted from the carrot discs by the maceration-sieving method (Speijer & De Waele, 1997).

4.2.4. FIRST EXPERIMENT: NEMATODE ATTRACTION AND PENETRATION

Six-weeks-old rooted tissue culture plantlets were planted in 200 ml cups filled with sand and potting soil (2:1). The plants were maintained under greenhouse conditions and fertilized at 10 days intervals throughout the experiment.

Six weeks after planting, approximately 1,000 living vermiform nematodes were inoculated per plant by pipetting 4 ml of the nematode suspension into inoculation holes made in the soil near the plant root zone. At 4, 8 and 12 days after inoculation, plants were uprooted and gently washed free of soil under running tap water. The fresh root and shoot weights were measured. The nematodes were extracted from the whole root systems by the maceration-sieving method (Speijer & De Waele, 1997) and counted as described in Chapter 2 (2.2.4). One replication of each genotype at every harvest time was stained with acid fuchsin to visualise and photo-document the nematodes that had penetrated the roots. All treatments (four genotypes at three harvest times) were replicated eight times and a randomized block design (RBD) was used. The experiment was repeated once to confirm the results.

4.2.5. SECOND EXPERIMENT: POST-INFECTIONAL NEMATODE DEVELOPMENT AND REPRODUCTION

Six-weeks-old rooted tissue culture plantlets were planted in 200 ml cups filled with sand and potting soil (2:1). The plants were maintained under greenhouse conditions and fertilized at 10 days intervals throughout the experiment.

Six weeks after planting, each plant was inoculated with a 4 ml suspension containing 1,000 living vermiform nematodes pipetted into inoculation holes made in the soil near the plant root zone. At 12 days after inoculation, plants were uprooted and gently washed free of soil under running tap water to limit nematode penetration to a maximum of 12 days. Then the plants were transplanted in 1 l pots filled with the sand and potting soil (2:1).

At 26 days after inoculation (*i.e.* 14 days after transplanting), plants were uprooted and gently washed free of soil under running tap water. The fresh root and shoot weights were measured. The nematodes were extracted from the whole root systems by the maceration-sieving method (Speijer & De Waele,

1997) and counted as described in Chapter 2 (2.2.4). The final nematode population densities were determined by calculating the sum of all developmental stages of the nematodes extracted from the whole root systems. The reproduction factor (Rf) was calculated as the final nematode population density divided by the inoculum. All treatments (four genotypes) were replicated eight times and a randomized block design was used.

4.2.6. STATISTICAL DATA ANALYSIS

Data with normal distribution and homogeneous variances were subjected to analysis of variances using STATISTICA version 9 (StatSoft, Tulsa, OK, USA). Nematode numbers were $\log_{10}(x+1)$ transformed prior to the analysis. When significant ($P \leq 0.05$) differences were observed, Tukey's HSD (Honestly Significant Difference) test was applied for multiple comparisons of group means.

4.3. RESULTS

4.3.1. FIRST EXPERIMENT: NEMATODE ATTRACTION AND PENETRATION

The fresh root and shoot weights of the *Musa* genotypes at 4, 8 and 12 days after inoculation (DAI) are presented in Table 4.1.

Lesions were observed on the secondary and tertiary feeder roots of all genotypes on 4 DAI. No visible damages were observed on the primary roots of the resistant and susceptible *Musa* genotypes at 4 DAI. Visibly large lesions were observed on the primary and secondary roots of Grande Naine at 8 and 12 DAI (Figs 4.1A & B). Much smaller lesions were visible on the primary roots of the resistant *Musa* genotypes at 8 and 12 DAI (Figs 4.1C & D).

The inoculated adults and juveniles had penetrated the roots of both resistant and susceptible *Musa* genotypes starting from the first observation at 4 DAI (Figs 4.2A & B). Eggs were observed next to the penetrated females in the roots of Grande Naine and Long Tavoy (Figs 4.2.C & D). Phenolic cells were observed in Yangambi km5 always at the nematode infection sites (Fig. 4.2E). Few nematodes were found invading the stele of Yangambi km5 especially in the secondary roots or at root junctions. No nematodes were observed in the stele of the other genotypes.

Table 4.1. Fresh root and shoot weights of the *Musa* genotypes at 4, 8 and 12 days after inoculation (DAI) with 1,000 adults and juveniles of *Radopholus similis*. The nematodes were inoculated around the roots of six-weeks-old plants. (n=8).

Genotypes	Root fresh weight (g)			Shoot fresh weight (g)		
	4 DAI	8 DAI	12 DAI	4 DAI	8 DAI	12 DAI
Long Tavoy	5.9a	7.4b	10.7b	20.1b	24.2b	25.7b
Saba	3.7a	5.4ab	8.1a	10.8a	17.4a	16.2a
Yangambi km5	4.5a	5.0ab	8.2ab	17.5b	18.2ab	20.6ab
Grande Naine	4.1a	4.1a	7.6a	16.3ab	17.2a	20.0ab

Means within a same column followed by the same letter are not significantly ($P \leq 0.05$) different according to Tukey's HSD test.

No significant differences were observed between the numbers of nematodes that had penetrated the four *Musa* genotypes at 4 and 8 DAI (Fig. 4.3). At 12 DAI, a significantly ($P \leq 0.05$) lower number of nematodes had penetrated the roots of Long Tavoy compared to Grande Naine. No significant differences were observed between the number of nematodes that had penetrated Saba, Yangambi km5 and Grande Naine at 12 DAI (Fig. 4.3). A decrease in the number of penetrated nematodes was observed in all genotypes at 12 DAI.

Eggs were laid by the females that had penetrated in Grande Naine at 8 DAI but not in any of the three resistant *Musa* genotypes at 8 DAI. At 12 DAI, eggs were also observed in the roots of Long Tavoy but the number of eggs was significantly ($P \leq 0.05$) lower than in Grande Naine. No eggs were observed in Saba and Yangambi km5 (Fig. 4.4).

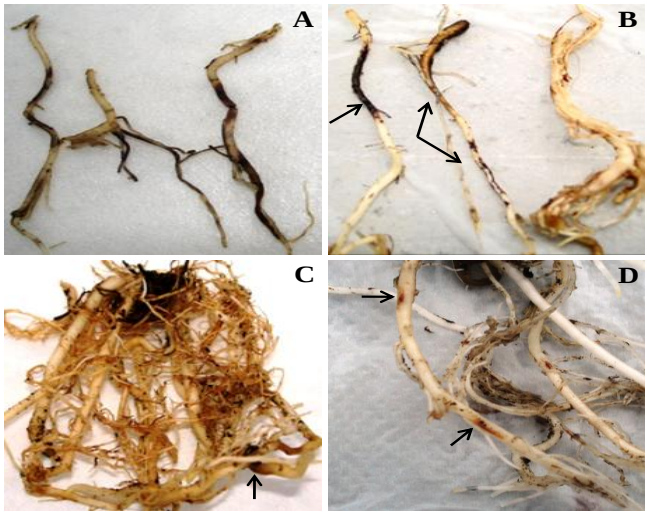


Figure 4.1: Root damage caused by *Radopholus similis*. A) and B) Lesions on the primary and secondary roots of Grande Naine at 8 days and 12 days after inoculation, respectively. C) Small lesions on the primary and secondary roots of Saba at 8 days after inoculation. D) Small lesions on the primary roots of Long Tavoy at 12 days after inoculation. Arrows indicate the lesions on the primary roots.

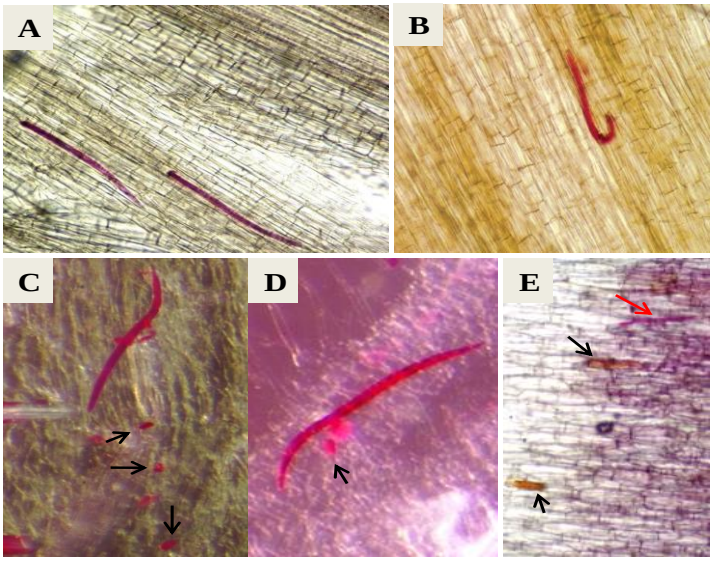


Figure 4.2: Females of *Radopholus similis* penetrating the roots of A) Grande Naine and B) Long Tavoy at 4 days after inoculation. Eggs (arrows) laid by the penetrated females at 12 days after inoculation in C) Grande Naine and D) Long Tavoy. E) Phenolic cells (black arrows) observed only around the nematode (red arrow) infection sites in Yangambi km5. Magnification: 40x

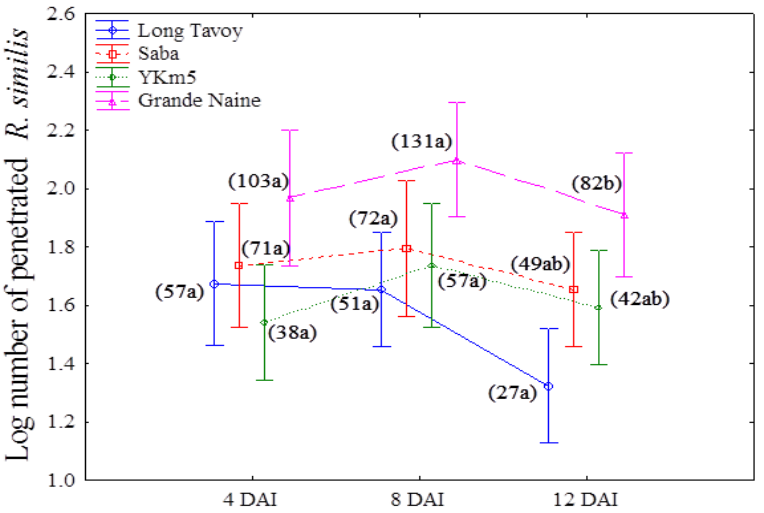


Figure 4.3: Number of *Radopholus similis* that had penetrated the roots of the resistant (Long Tavoy, Saba, Yangambi km5) and the susceptible (Grande Naine) *Musa* genotypes at 4, 8 and 12 days after inoculation with 1,000 adults and juveniles of *Radopholus similis*. Data were $\log_{10}(x+1)$ transformed prior to statistical analysis. The original means are presented between parentheses. Means within a same time (DAI) followed by the same letter are not significantly ($P \leq 0.05$) different from each other according to Tukey's HSD test. Error bars represent confidence intervals. (n=8).

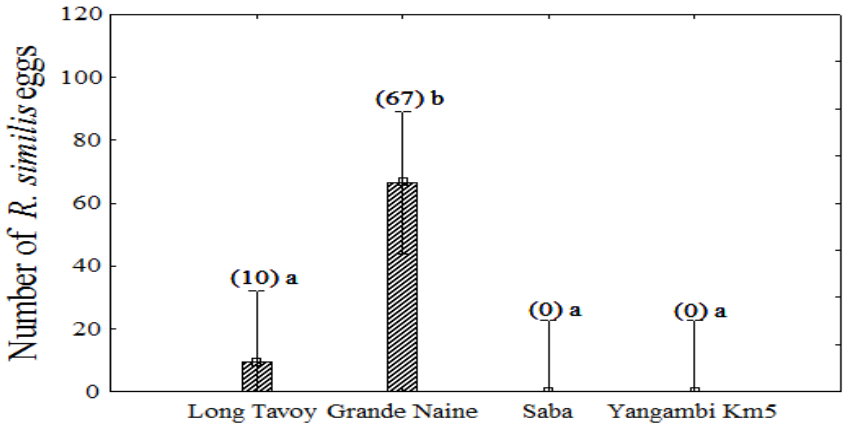


Figure 4.4: Number of *Radopholus similis* eggs observed in the roots of the resistant (Long Tavoy, Saba, Yangambi km5) and the susceptible (Grande Naine) *Musa* genotypes at 12 days after inoculation with 1,000 adults and juveniles of *Radopholus similis*. Means are presented between parentheses. Means followed by the same letter are not significantly ($P \leq 0.05$) different from each other according to Tukey's HSD test. Error bars represent confidence intervals. (n=8).

4.3.2. SECOND EXPERIMENT: NEMATODE DEVELOPMENT AND REPRODUCTION

The fresh root and shoot weights of the *Musa* genotypes at 26 days after inoculation are presented in Table 4.2. Long Tavoy had the highest root and shoot weights while Grande Naine had the lowest root and shoot weights.

Table 4.2. Fresh root and shoot weights of the *Musa* genotypes at 26 days after inoculation (DAI) with 1,000 adults and juveniles of *Radopholus similis*. The nematodes were inoculated around the roots of six-weeks-old plants. (n=8).

Genotypes	Root fresh weight (g)	Shoot fresh weight (g)
Long Tavoy	20 a	50 a
Saba	15 b	28 b
Yangambi km5	13 b	39 ab
Grande Naine	13 b	33 b

Means within a same column followed by the same letter are not significantly ($P \leq 0.05$) different from each other according to Tukey's HSD test.

Nematode development and reproduction were significantly ($P \leq 0.05$) different among the four genotypes. In Grande Naine, the number of eggs, males, females, number of adults and juveniles (excluding the eggs), and final population density were significantly ($P \leq 0.05$) higher than in the three other *Musa* genotypes. Grande Naine had the highest reproduction factor while Yangambi km5 had the lowest reproduction factor (Table 4.3 & Fig. 4.5)

The proportion of different developmental stages to the total population shows that a high proportion of eggs was present in Grande Naine compared to the resistant genotypes. In Yangambi km5, the proportion of females is somewhat higher compared to the three other genotypes. Apart from these two differences, the trend of population ratios looks similar in all the genotypes. The number of eggs and final nematode population density were about 15 and 10 times, respectively, lower in Yangambi km5 than in Grande Naine (Table 4.3).

Table 4.3. Number of *Radopholus similis* eggs, juveniles, females, males, final population density and reproduction factor (Rf) in the roots of the resistant (Long Tavoy, Saba, Yangambi km5) and the susceptible (Grande Naine) *Musa* genotypes at 26 days after inoculation with 1,000 adults and juveniles of *Radopholus similis*. (n=8).

Genotype	Mean number of *Radopholus similis*/root system					
	Eggs	Juveniles	Females	Males	Final population density	Rf
Long Tavoy	129 b	187 b	246 b	6 a	568 b	0.57
Saba	99 b	210 b	238 b	3 a	549 b	0.55
Yangambi km5	38 a	47 a	101 a	1 a	188 a	0.19
Grande Naine	555 c	480 b	820 c	34 b	1,890 c	1.89

Means within a same column followed by the same letters are not significantly ($P \leq 0.05$) different from each other according to Tukey's HSD test. Rf: reproduction factor = final nematode population density/initial inoculum density.

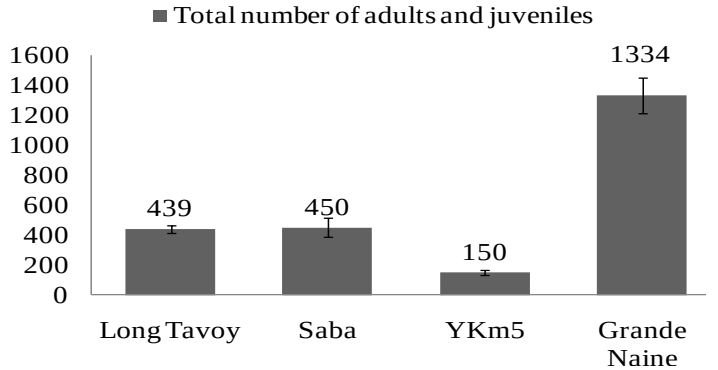


Figure 4.5: Number of adult and juvenile *Radopholus similis* in the roots of resistant (Long Tavoy, Saba, Yangambi km5) and susceptible (Grande Naine) *Musa* genotypes at 26 days after inoculation with 1,000 adults and juveniles of *Radopholus similis*. Error bars represent standard error. (n=8).

4.4. DISCUSSION

This part of our study clearly demonstrates that the three *R. similis*-resistant *Musa* genotypes impair the post-infectional development and reproduction of *R. similis*. The pre-infectional resistance factors, on the other hand, do not impair the nematodes' attraction towards and penetration in the roots. No significant differences were observed in the number of nematodes that had penetrated the resistant and susceptible *Musa* genotypes until 8 days after inoculation (DAI).

In a previous study, Mateille (1992) found that the penetration of *R. similis* was initially slower in the roots of the resistant *Musa* genotype Gros Michel than in the susceptible *Musa* genotype Poyo. However, 2 weeks later, an equal

number of nematodes had invaded both the resistant and susceptible *Musa* genotype. No differences were observed in the penetration of *R. similis* in Yangambi km5 and French Sombre, a susceptible plantain (Fogain, 2000b). Similarly, no differences were reported in the penetration of *R. similis* in resistant and susceptible *Musa* cultivars based on an *in vitro* study using excised roots (Dochez, 2004).

In contrast, significant differences were observed in the penetration of *R. similis* in the susceptible *Musa* genotype Poyo and Yangambi km5 at 24 h after inoculation in an *in vitro* study (Valette *et al*., 1997). In our study, *R. similis* penetration was lower in only one resistant *Musa* genotype, Long Tavoy, than in Grande Naine at 12 DAI. However, already at 8 DAI the second generation of nematodes (eggs) were observed in Grande Naine as the inoculum consisted mostly of gravid females. This could have influenced the higher number of nematodes observed in Grande Naine at 12 DAI. The second generation did not appear in Yangambi km5 and Saba until 12 DAI. Though eggs were found in Long Tavoy at 12 DAI, the number was significantly lower than the number of eggs found in Grande Naine.

In our study, few nematodes invaded the stele of Yangambi km5 especially in the secondary roots or at root junctions. This might have been due to the absence of lignifications in the young roots. A previous study reported the invasion of the vascular region by *R. similis* in the susceptible *Musa* genotype Poyo (Valette *et al*., 1997).

Results from our nematode development and reproduction experiment showed a significant increase in the numbers of eggs, adults and the final nematode population density in Grande Naine compared to all three resistant *Musa* genotypes at 26 DAI. This shows that the resistance mechanisms of the *R. similis*-resistant *Musa* genotypes examined are strongly influencing the post-infectional nematode development and reproduction of *R. similis*. In a previous study, the resistant *Musa* genotype Gros Michel significantly reduced the reproduction factor of *R. similis* compared to the susceptible *Musa* genotype Poyo at 2 months after inoculation (Mateille, 1992). Investigations on the resistance mechanisms against the migratory endoparasite *R. similis* are scarce. Investigations on the sedentary endoparasitic root-knot nematodes (*Meloidogyne* species) showed that initial root penetration by *Meloidogyne incognita* was not impaired in resistant genotypes of cotton (Faske & Starr, 2009) and soybean (Fourie, 2005). *Meloidogyne arenaria*, *M. incognita*, *M. javanica* and *M. hapla* readily penetrated resistant and susceptible cucumber plants leading to the conclusion that reduced nematode development and

reproduction are clearly one of the mechanisms of resistance involved (Walters *et al*., 2006).

Based on the many reported investigations on sedentary and some migratory endoparasitic nematodes, it was stated that these nematodes freely penetrate the roots of resistant and susceptible plants and that incompatibility at this stage may occur but is rare. Preformed morphological and chemical barriers do not appear to be effective against penetration of plant tissues by these nematodes (Kaplan & Keen, 1980). Published reports showed that many resistance host responses, particularly those involving biotrophic pathogens, are governed by specific interactions between a pathogen avirulent gene and a plant resistance gene (Dangl & Jones, 2001) and less due to preformed factors. Moreover, most of the identified and cloned nematode resistance genes act *via* a localized necrosis or hypersensitive response influencing the post-infectional migration, development and reproduction of the nematodes (Williamson & Hussey, 1996; Das *et al*., 2008).

4.5. CONCLUSION

No significant differences were observed in the number of *R. similis* penetrating the resistant and susceptible *Musa* genotypes included in our study except in the resistant genotype Long Tavoy at 12 days after inoculation. Post-infectional nematode development and reproduction were impaired in the resistant *Musa* genotypes. On the basis of these observations it appears that the mechanism of resistance in the investigated *Musa* genotypes to *R. similis* is induced after nematode penetration and that preformed host resistance factors do not function strongly against the nematode attraction and migration towards the roots, and penetration of the roots.

CHAPTER 5

LIGNIN AND PHENOLS INVOLVED IN THE INTERACTIONS BETWEEN *RADOPHOLUS SIMILIS* AND *MUSA* SPP.

5.1. INTRODUCTION

Plants produce more than 100,000 diverse, low molecular weight secondary metabolites (Dixon, 2001). Secondary metabolites play a major role in the plants' interaction with the environment ensuring successful adaptation and survival (Verpoorte, 2000). Phenolic secondary metabolites such as lignin and phenols were associated with nematode resistance in bananas (Valette *et al*., 1998; Wuyts *et al*., 2007).

Lignin is the second most abundant natural biopolymer after cellulose, found in all vascular plants (Ferrer *et al*., 2008). Lignin is estimated to comprise about 20% of the global biomass (Chen *et al*., 2000). Lignin deposition strengthens the cell walls. The plant cell wall is vital for the structural integrity of plants, plant growth, intercellular communication, water movement and pathogen defense (Aquije *et al*., 2010).

The resistance of lignin to microbial degradation was thought to provide a hard barrier to invading pathogens (Tronchet *et al*., 2010). Presence of a higher number of lignified cell walls in the vascular bundles of the *Musa* spp. cv. Pisang Jari Buaya was associated with its resistance to *Radopholus similis* (Fogain & Gowen, 1996). Lower root dry matter was correlated with lower lignin content resulting in the susceptibility of the *Musa* spp. cv. Poyo to *R. similis* (Fogain & Gowen, 1996). Higher levels of constitutive lignin contents were observed in *R. similis*-resistant *Musa* cultivars than in the susceptible cultivar Grande Naine (Wuyts *et al*., 2007).

A higher number of preformed phenolic cells was related to the resistance of *Musa* spp. cv. Yangambi km5 to *R. similis* (Fogain & Gowen, 1996). Constitutive presence of lignin, dopamine, flavonoids, caffeic and ferulic acids in Yangambi km5 roots was associated with the resistance to *R. similis* and hypothesized that they act as chemical barriers for *R. similis* penetration and colonization (Valette *et al*., 1998).

In this part of our study, preliminary phytochemical studies were carried out to assess the involvement of lignin and phenols in the resistance of the newly identified *R. similis*-resistant *Musa* genotypes Long Tavoy (*Musa acuminate* AA, Burmannica subgroup, ITC 0283) and Saba (*Musa* spp. ABB, Saba subgroup, ITC 1138). The well known reference cultivars Yangambi km5 (*Musa acuminata* AAA, Ibota subgroup, ITC 1123) (resistant) and Grande Naine (*Musa acuminata* AAA, Cavendish subgroup, ITC 1256) (susceptible) were also included in the study as control. The lignin and total phenols were localised by histochemical staining in root cross sections of *R. similis*-infected and uninfected plants. Quantitative assessments were performed on the root

extracts of all the plants. The results are discussed comparing *R. similis*-infected and uninfected plants of susceptible and resistant *Musa* genotypes.

5.2. MATERIALS AND METHODS

5.2.1. PLANTING MATERIAL

All *Musa* genotypes were initially obtained from the *Musa* germplasm collection maintained at the International Transit Centre (ITC), K.U.Leuven, Belgium. The plant material was proliferated, regenerated and rooted in test tubes as explained in Chapter 2 (2.2.2). The plantlets were grown in growth chambers at 28 °C and 16 h photoperiod.

5.2.2. NEMATODE INOCULUM

A population of *R. similis* originally isolated from banana roots in Uganda was used in the experiments. This population was maintained and multiplied monoxenically on sterile carrot discs at 25±1 °C in the dark (Speijer & De Waele, 1997). The population from Uganda was characterized by a high reproductive fitness (Fallas *et al*., 1995). To obtain the inoculum, *R. similis* was extracted from the carrot discs by the maceration-sieving technique (Speijer & De Waele, 1997).

5.2.3. EXPERIMENTAL SET-UP

Six-weeks-old rooted tissue culture plantlets were planted in 1 l pots filled with sand and potting soil (2:1). Eight weeks after planting, two sets of eight plants of each *Musa* genotype were inoculated with approximately 1,000 living vermiform nematodes by pipetting 4 ml of the nematode suspension into inoculation holes made in the soil near the plant root zone. Two sets of eight uninfected plants of each *Musa* genotype were included as control plants. The plants were placed in the greenhouse according to a randomized block design.

The plants were maintained under greenhouse conditions and fertilized at 10 days intervals throughout the experiment. In the greenhouse, the ambient day and night temperatures were 27 and 20 ºC, respectively, relative humidity was 80% and the photoperiod was 12 h.

5.2.4. SAMPLING AND ASSESSMENT OF NEMATODE INFECTION

One set of eight *R. similis*-infected and one set of eight uninfected plants were uprooted at 3 weeks after inoculation. The second sets were harvested at 6

weeks after inoculation. At both sampling times, the root systems were carefully washed free of soil (without destroying the root epidermis) under running tap water. The fresh root and shoot weights of the whole root systems were measured. About half of the fresh roots were used for histochemical staining and nematode extraction. The remaining roots were snap frozen in liquid nitrogen and stored at -80 ºC for quantitative assays of lignin and total phenols. Necrotic cells from *R. similis*-infected roots (~0.5 g) were manually dissected under a stereoscopic microscope for nematode extraction. Nematodes were extracted from the micro-dissected lesions by opening the roots with a needle in a thin film of water and the nematodes were counted under a stereoscopic microscope.

5.2.5. HISTOCHEMICAL STAINING OF ROOT CROSS SECTIONS

Thin, free hand-cut root cross sections of *R. similis*-infected and uninfected *Musa* genotypes were used for the histological staining of lignin and total phenols. The Wiesner reagent was used for the localisation of lignin (syringyl and guaiacyl units) and Mäule reagent was used for the localisation of the syringyl units of lignin.

For Wiesner staining, hand-cut root cross sections were fixed in 2% phloroglucinol in ethanol/water (95/5, v/v) for 10 min and mounted in 37% HCl on glass slides. Wiesner reagent reacts with cinnamaldehyde end groups in the lignin resulting in the cationic chromophore which appears as a burgundy-red compound (Vermerris & Nicholson, 2006).

For Mäule's staining, hand-cut root cross sections were immersed in 1% $KMnO_4$ for 30 min, rinsed with distilled water and destained using 20% HCl for 2 min. The stained sections were mounted in 10% NH_3 on glass slides. The presence of syringyl units results in the development of a deep red colour while the absence of syringyl units results in the development of a yellow colour (Vermerris & Nicholson, 2006).

For the localisation of total phenols, fresh root cross sections were treated with 0.5% (v/v) toluidine blue in 2.5% Na_2CO_3 at pH 9 for 10 min and rinsed with 70% ethanol. Stained root sections were observed under a bright field transmitted light microscope. Phenolic cells stain as deep blue (Valette *et al*., 1997).

Fluorescent flavonoids and lignified cell walls were detected by Neu's reagent (Valette *et al*., 1998). Fresh root cross sections were stained with freshly prepared 0.25% (w/v) diphenylboric acid 2-aminoethyl ester (DPBA) in MilliQ water with 0.02% (v/v) triton-x-100 for 2 min. Sections were observed

immediately under an epifluorescence microscope with a DAPI filter (excitation 340-380 nm, suppression LP 430 nm) and a FITC filter (excitation 450-490 nm, suppression LP 520 nm). Photography of the root sections was done using a SPOT RT CCD camera and SPOT RT software version 3.5.5 (Diagnostic Instruments, Inc., USA)

5.2.6. EXTRACTION AND QUANTIFICATION OF LIGNIN

Lignin contents of isolated cell wall samples were determined by using the thioglycolic acid derivatised lignin method as described by Lange *et al*. (1995). To isolate the cell walls, 500 mg of frozen root samples were ground in liquid nitrogen using a mortar and pestle without letting the sample thaw. Ground root samples were first suspended in 1.5 ml methanol and vigorously stirred for 1 h. The suspension was centrifuged for 5 min at 16,000 x g. Pellets were suspended in 1.5 ml of the following seven solutions one after the other by vigorously stirring for 15 min and each time the suspension was centrifuged for 5 min at 16,000 x g: (a) methanol, (b) 1 M NaCl, (c) 1% SDS (w/v), (d) twice with MilliQ water, (e) ethanol, (f) chloroform/methanol (1:1, v/v) and (g) tert-butyl methyl ether. The resultant pellets are the purified cell walls. The purified cell wall pellets were freeze-dried overnight.

Approximately 15 mg of the cell wall preparations were taken and suspended in a mixture of 1 ml HCl (2 M) and 200 µl thioglycolic acid. Suspensions were incubated for 4 h in a water bath at 95$\pm$2 ºC. After cooling down to room temperature, samples were centrifuged for 10 min at 16,000 x g. Pellets were washed three times with MilliQ water, suspended in 1 ml NaOH (0.5 M) and vigorously shaken overnight to extract the lignothioglycolic acid (LTGA). Samples were centrifuged for 10 min at 16,000 x g and the supernatants were collected. The pellets were washed with an additional 500 µl of NaOH (0.5 M). The combined supernatants (alkali extracts) were acidified with 300 µl concentrated HCl and incubated for 4 h at 4 ºC to precipitate the LTGA from the alkali extracts. Samples were centrifuged for 10 min at 16,000 x g and pellets were dried in a SpeedVac centrifuge. Dry brown pellets were dissolved in 1 ml NaOH (0.5 M) to measure the absorbance.

Absorbance of the samples was measured against the blank at 280 nm in a NanoDrop spectrophotometer using the UV-Vis high absorbance mode (ND-1000, NanoDrop Technologies, Inc., Wilmington, USA). The lignin content (LTGA) was expressed as mg lignin per g fresh root weight using a calibration curve of alkali lignin (Sigma-Aldrich, Inc., Borem, Belgium).

5.2.7. FOLIN-CIOCALTEU ASSAY FOR TOTAL PHENOLICS

This method determines the total soluble phenolic content based on the reduction of the phospho-molybdene/phospho-tungstate present in the Folin–Ciocalteu reagent. One g of frozen root samples were ground in liquid nitrogen using a mortar and pestle without letting the sample thaw. Ground root samples were extracted with 5 ml of 50% methanol in test tubes and secured with screw caps. Samples were shaken at 4 ºC for 60 min. Each extract was passed through a 0.45 µm PTFE filter (Merck) and the filtrate was kept on ice. For the analysis of total phenols using the Folin-Ciocalteu method (Makkar *et al.*, 1993), 100 µl of the extract was mixed with 400 µl of distilled water in glass test tubes. To each of the samples 250 µl of 1 N Folin-Ciocalteu reagent and 1,250 µl of 20% sodium carbonate solution were added and vortexed. The samples were kept in the dark at room temperature for 40 min. Absorbance was measured at 725 nm against a blank using a Novaspec II spectrophotometer. A standard calibration curve of tannic acid was established and total phenolic content was expressed as tannic acid equivalents per g of fresh root weight (TAE/g FRW).

5.2.8. STATISTICAL ANALYSIS

Statistical analysis was performed using STATISTICA version 9 (StatSoft, Tulsa, OK, USA). Data on fresh root and shoot weights and lignin content were analysed using analysis of variance (ANOVA). When significant differences ($P \leq 0.05$) were observed, Tukey's HSD test was applied for multiple comparisons of group means. Nematode numbers and total phenolic content data were analysed using Kruskal-Wallis analysis of variance by ranks. The non parametric equivalent of ANOVA, Kruskal-Wallis analysis of variance by ranks, was applied because the data set did not meet the basic assumptions of the parametric tests namely normal distribution and homogeneous variances. When the Kruskal-Wallis analysis of variance by ranks was significant, multiple comparisons between treatments were calculated as described by Siegel and Castellan (1988).

5.3. RESULTS

5.3.1. ROOT AND SHOOT WEIGHT

The effect of *R. similis* infection on the root and shoot fresh weights of the *Musa* genotypes are summarised in Table 5.1.

Table 5.1. Mean fresh root and shoot weights of *Radopholus similis*-infected and uninfected *Musa* genotypes at 3 and 6 weeks after inoculation with 1,000 adults and juveniles of *R. similis.*

Treatments	Saba	Long Tavoy	Yangambi km5	Grande Naine
Root fresh weight (g)				
3 weeks				
+ *R. similis*	33.8*	44.4*	32.1	17.3
- *R. similis*	33.3	38.4*	28.7	19.5
6 weeks				
+ *R. similis*	53.4*	64.0*	69.5†*	34.7
- *R. similis*	51.7	55.2*	46.1†	37.0
Shoot fresh weight (g)				
3 weeks				
+ *R. similis*	88.1*	107.8*	91.0*	51.6
- *R. similis*	87.1*	113.1*	83.0	58.5
6 weeks				
+ *R. similis*	114.0*	131.8*	116.2*	73.4
- *R. similis*	113.3*	116.7*	102.7	79.0

Means followed by † indicates significant differences ($P \leq 0.05$) between the *R. similis*-infected and uninfected plants of the same genotype within the same time (3 or 6 weeks). Means followed by * indicates significant differences ($P \leq 0.05$) compared to the mean shoot or root weight of the susceptible reference genotype Grande Naine in the same row. Significance of the differences was tested using the Tukey's HSD test.

Root weights of infected Yangambi km5 plants were significantly ($P \leq 0.05$) higher than the root weights of the uninfected plants of the same genotype at 6 weeks after infection. No significant differences in root weight between infected and uninfected plants were observed for the three other *Musa* genotypes. No significant differences in shoot weight between infected and uninfected plants were observed. The fresh root and shoot weights of Grande Naine were always lowest compared with the other three *Musa* genotypes.

5.3.2. ROOT ANATOMY AND CELLULAR DAMAGE IN INFECTED ROOTS

Thin, fresh, hand-cut root cross sections were made from all the *Musa* genotypes to compare the anatomical structures of the uninfected and *R. similis*-infected plants. In the infected plants, nematode damage and lesion patterns were studied. The anatomical root structures in the uninfected *Musa* root cross sections are shown in Figure 5.1. The anatomy of the healthy roots was similar to previous descriptions of *Musa* roots (Acquarone, 1930; Wuyts, 2006).

The outermost layer is the root epidermis lining the root cortex. Few outermost cortical layers are thick cork cells. The outer cortex layers are composed of large cells, arranged in irregular radial rows with small intercellular spaces. The inner side of the outer cortex continues to form aerenchyma previously described as lacunae by Acquarone (1930). The aerenchyma is wide and the cavities are lined by radial cortical cell plates. The inner cortex follows the aerenchyma internally. The inner cortical cells are smaller, cylindrical and arranged in more regular radial rows with well defined larger intercellular spaces (Fig. 5.1A). The inner cortical cells become smaller for every inner row. The innermost cortex layer is a layer of very small, well packed cells called endodermis. Suberin deposition thickens the lateral sides of endodermal cells.

The pericycle is the outermost layer of the vascular bundle made of a single layer of cells lying just below the endodermis. As a monocotyledon root, the vascular bundle is a polyarch type and radial in arrangement. The outer protoxylem vessels lie against the pericycle. The metaxylem vessels are strikingly large. Each xylem vessel is lined by a layer of sclerenchyma (wood parenchyma). The peripheral and central phloem strands are found in between the xylem vessels (Fig. 5.1B).

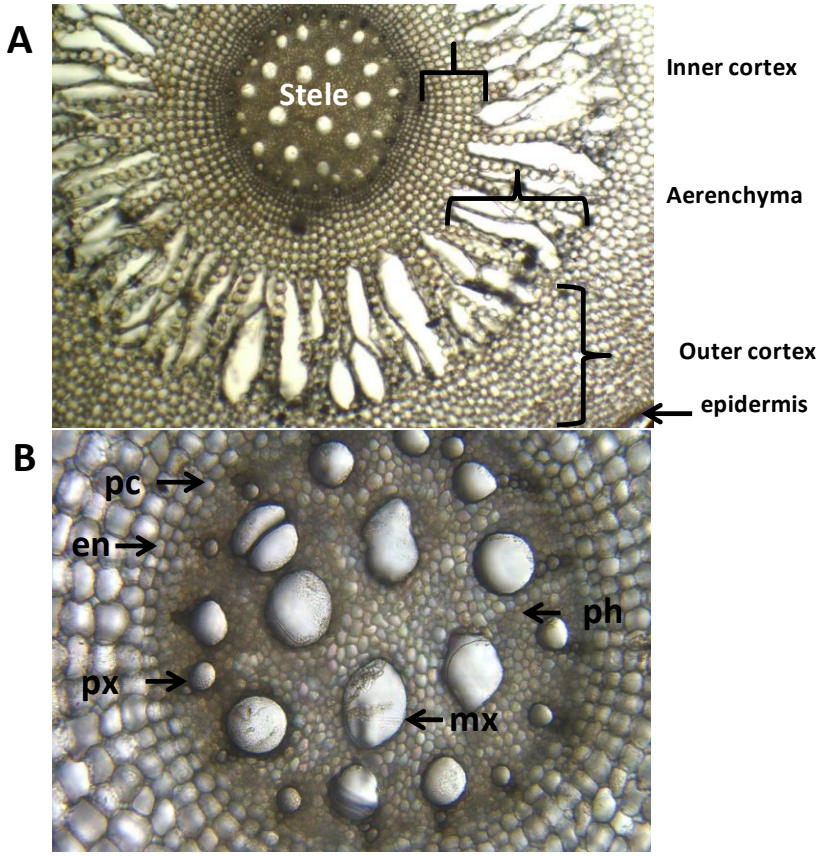


Figure 5.1: Anatomical root structures of *Musa* spp. cv. Saba (cross sections). A) Epidermis, inner and outer cortex, aerenchyma and vascular bundle (stele) (x40). B) The vascular bundle with its different structures (x100); en: endodermis; mx: metaxylem; pc: pericycle; ph: phloem cells; px: protoxylem.

The necrotic lesions in *R. similis*-infected root cross sections were observed under a compound, transmitted light microscope. The images of the lesions are presented in Figure 5.2. The necrosis started from the epidermis and extended into the outer cortex, aerenchyma and inner cortex but it was more extensive in the aerenchyma of all the four *Musa* genotypes (Fig. 5.2C). Necrosis was also observed in the endodermis, extending into the outer layers of vascular bundle of Yangambi km5 at 3 weeks after infection. Observation of the lesions showed that the cells were dark brown coloured in Yangambi km5 and Saba (Figs 5.2B, E & F) and reddish brown coloured in Grande Naine (Fig. 5.2A).

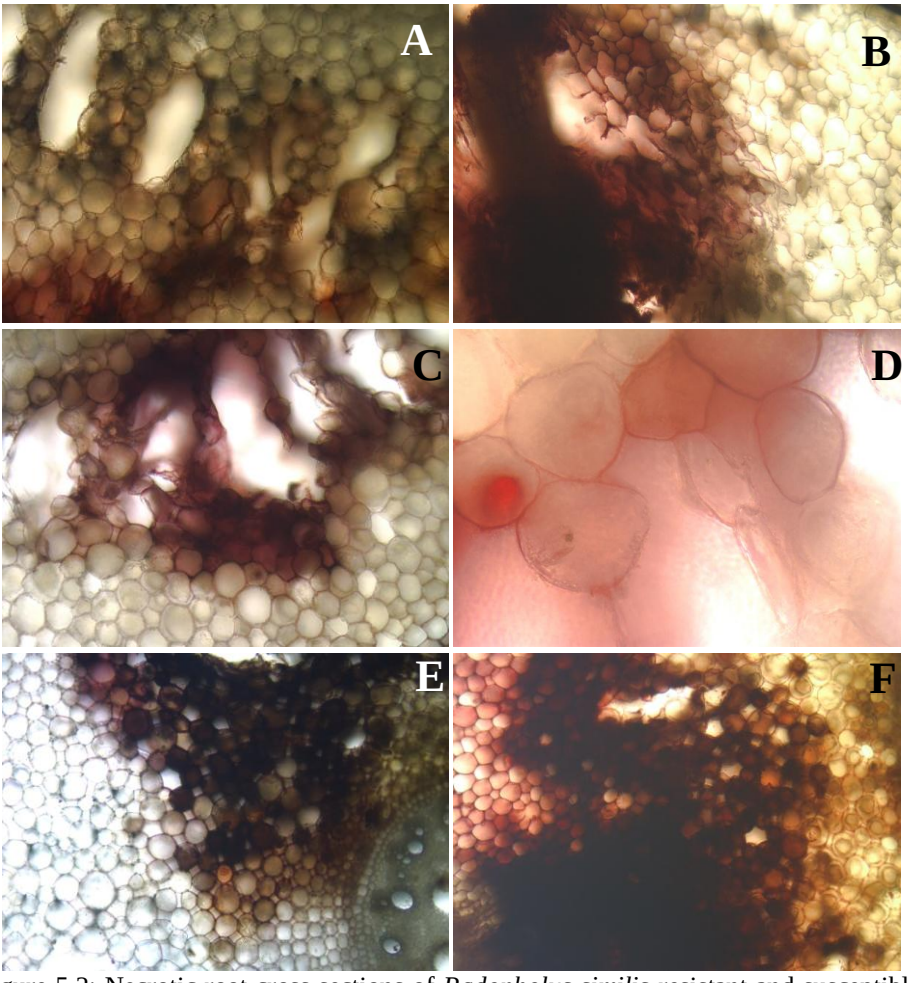


Figure 5.2: Necrotic root cross sections of *Radopholus similis*-resistant and susceptible *Musa* genotypes at 6 weeks after inoculation with *R. similis*.
A) Young lesions extending from the cortex to the aerenchyma cells of Grande Naine. B) Dark brown necrosis in the cortex of Saba. C) Lesions in the aerenchyma cells of Long Tavoy. D) Phenolic compounds starting to accumulate in a newly developing lesion in Long Tavoy. E) Developing lesions with high phenol contents (dark brown cells) in the necrotic areas of Yangambi km5. F) Well developed lesions in Yangambi km5. Magnification of the images except D: 100x; D: 400x

5.3.3. NEMATODE NUMBERS IN NECROTIC LESIONS

The numbers of *R. similis* in the necrotic lesions of the resistant and susceptible *Musa* genotypes at 6 weeks after infection are summarised in Table 5.2. The mean number of nematodes extracted from the necrotic lesions of susceptible *Musa* genotype Grande Naine was significantly ($P \leq 0.05$) higher

than the number of nematodes extracted from the resistant *Musa* genotypes Saba, Long Tavoy and Yangambi km5.

Table 5.2. Mean number of *Radopholus similis* in the necrotic lesions of three resistant (Saba, Long Tavoy & Yangambi km5) and one susceptible (Grande Naine) *Musa* genotypes at 6 weeks after inoculation with 1,000 adults and juveniles of *R. similis* (n=4).

Genotype	Nematodes/g of necrotic lesion
Saba	583 a
Long Tavoy	254 a
Yangambi km5	255 a
Grande Naine	1057 b

Means within the same column followed by the same letter are not significantly ($P \leq 0.05$) different from each other according to the Kruskal-Wallis analysis of variance by ranks.

5.3.4. HISTOCHEMICAL STAINING OF *MUSA* ROOT CROSS SECTIONS FOR LIGNIFIED CELL WALLS

Lignified cell walls fluoresce under UV light when stained with DPBA. When the root section is viewed through a FITC filter, the syringyl units fluoresce in green and the guaiacyl units fluoresce in bright yellow colour. The uninfected plants of all *Musa* genotypes sampled at 6 weeks showed intense fluorescence (Figs 5.3B, D, F, G & I) compared to the plants sampled at 3 weeks after infection (Figs 5.3A, C, E & H) *i.e.* older roots showed more extensive secondary wall thickening due to lignin deposition. The uninfected plants of the *R. similis*-resistant genotype Saba showed distinct lignification of aerenchyma with syringyl units (Fig. 5.3D).

Radopholus similis-infected plants (Fig. 5.4) showed much intense fluorescence in the vascular bundles and endodermis compared to the uninfected plants of the same age (Fig. 5.3). The *R. similis*-infected plants of Long Tavoy showed the most extensive secondary wall lignification of vascular parenchyma (sclerenchyma) cells (Figs 5.4I & J) compared to all other genotypes. Lignification starts at the endodermis (Fig. 5.4E), extending to the peripheral tissues of vascular system especially the xylem walls, xylem-accompanying parenchyma cells (Fig. 5.4H) and eventually the lignification progress to the vascular parenchyma (sclerenchyma) cells in the centre (Figs 5.4I & J).

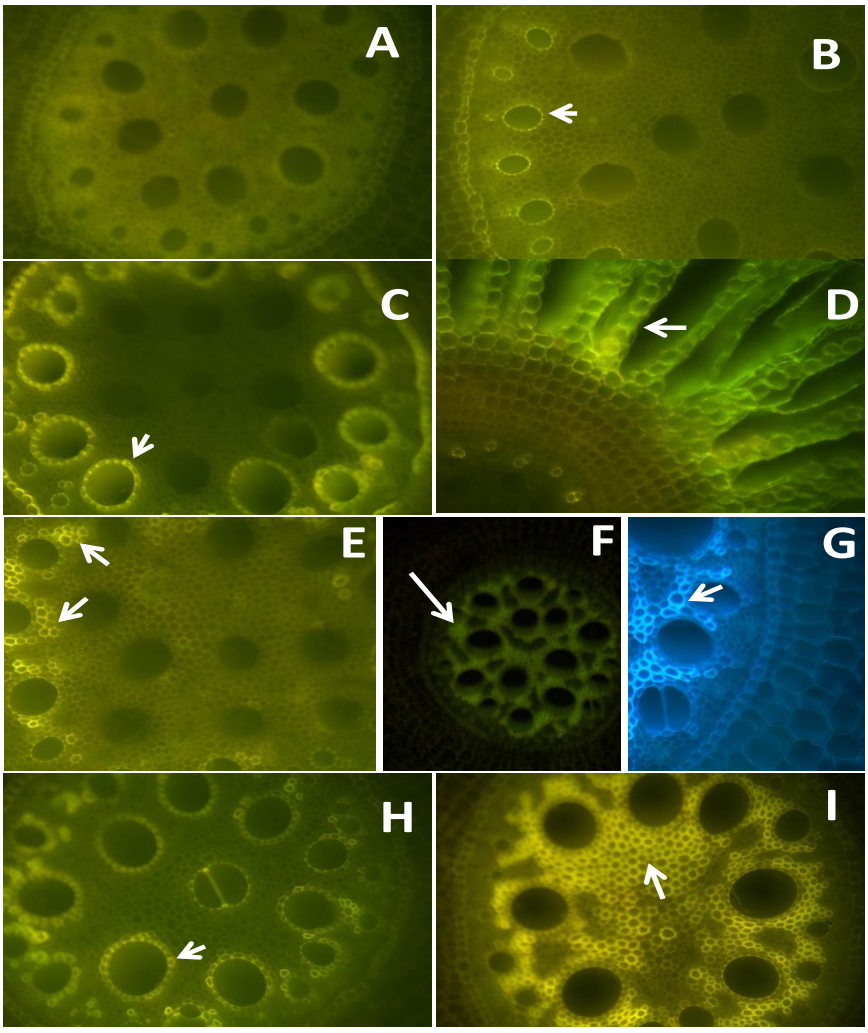


Figure 5.3: Lignifications in uninfected *Musa* root cells at 3 and 6 weeks.
Fresh, hand-cut root cross sections were stained with DPBA and observed under epifluorescence microscope using FITC (all images except G) and DAPI filter (G). A) Vascular bundle of Grande Naine at 3 weeks showing no cell wall lignification and B) initial lignification of protoxylem cells of Grande Naine at 6 weeks. C) Initial lignifications in the xylem-accompanying vascular parenchyma cells of Saba at 3 weeks and D) lignified aerenchymatic cells at 6 weeks in Saba. E) Initial lignifications of parenchyma cells surrounding xylem cells of Long Tavoy at 3 weeks and F) & G) lignified vascular cell (sclerenchyma) walls of Long Tavoy at 6 weeks. H) Initial lignifications of xylem cells of Yangambi km5 at 3 weeks and I) strong lignifications progressing to the centre of the vascular bundles of Yangambi km5 at 6 weeks. Arrows are pointing at the lignified cell walls. Magnification of the images: 100x.

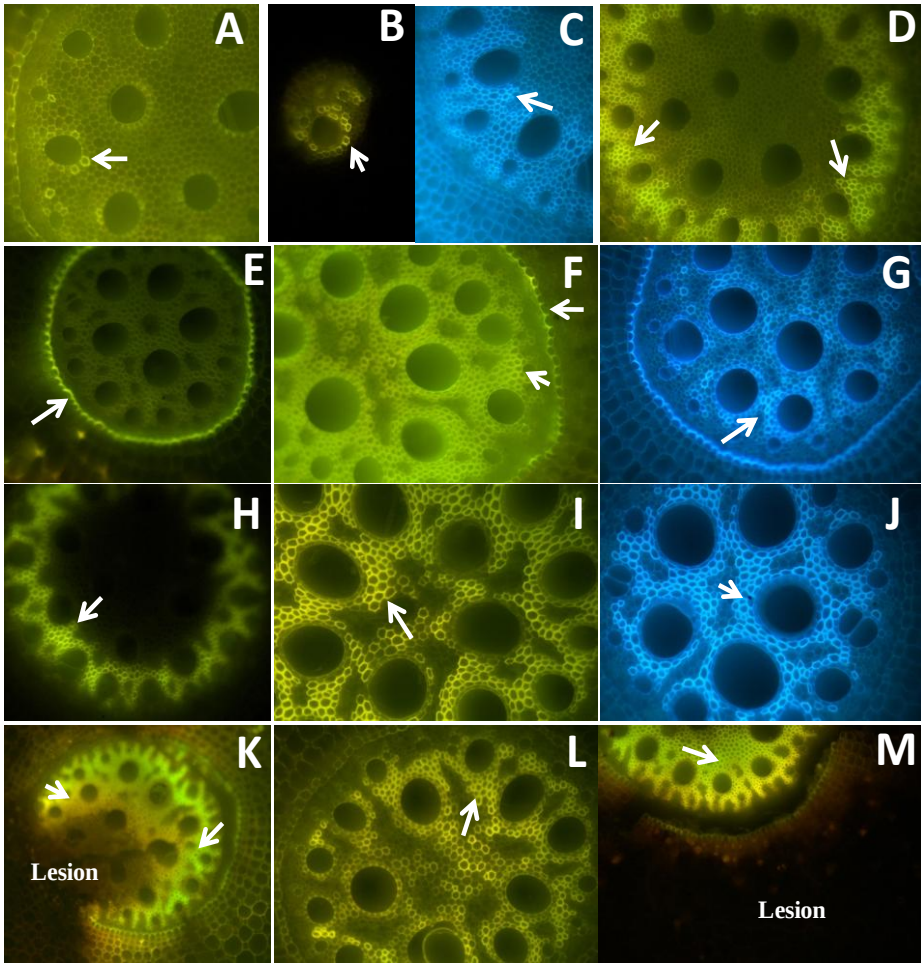


Figure 5.4: Lignifications in *Radopholus similis*-infected *Musa* root cells at 3 and 6 weeks after inoculation with 1,000 adults and juveniles of *R. similis*

Fresh, hand-cut root cross sections were stained with DPBA and observed under epifluorescence microscope using FITC (except C, G & J) and DAPI filter (C, G & J). A) & B) Initial lignifications of parenchyma cells surrounding protoxylem of Grande Naine at 3 weeks. C) & D) Extended lignifications of vascular bundles of Grande Naine at 6 weeks. E) Localisation of syringyl lignin (arrow) in the endodermis of Saba roots at 3 weeks. F) & G) Lignifications in the xylem accompanying parenchyma cells of Saba at 6 weeks. H) Intense syringyl lignifications (arrows) in the peripheral vascular cells of Long Tavoy at 3 weeks. I) & J) Intense guaiacyl lignification in the vascular parenchyma cells of Long Tavoy at 6 weeks. K) Intense lignifications in sclerenchyma of Yangambi km5 at 3 weeks when the nematode damage extended into vascular bundles. L) Guaiacyl lignin deposition in the vascular sclerenchyma of Yangambi km5 at 6 weeks. M) Intense deposition of syringyl (greenish) and guaiacyl (yellowish fluorescence) lignifications in Yangambi km5 vascular cells against a lesion at 6 weeks. Arrows are pointing at the lignified cell walls. Magnification of the images: 100x.

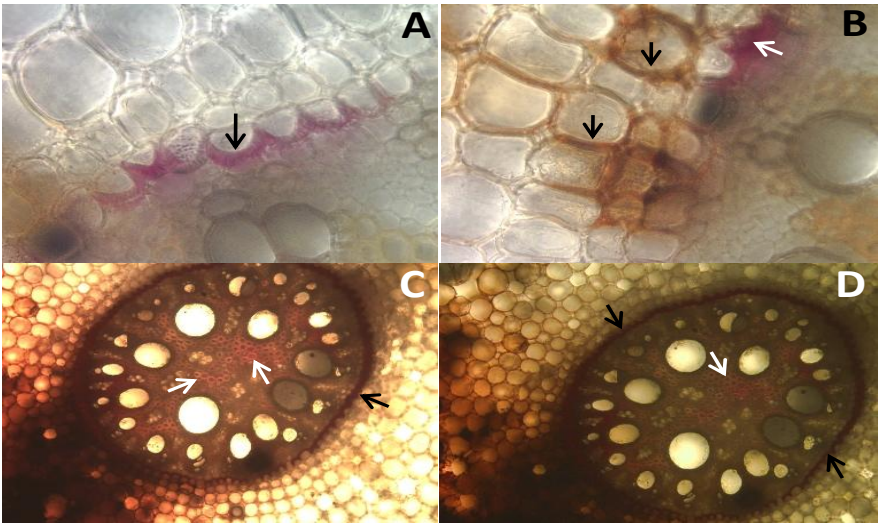


Figure 5.5: Tissue localisation of lignin in the root cross sections of *Radopholus similis*-resistant *Musa* genotype Yangambi km5 at 6 weeks after inoculation with 1,000 adults and juveniles of *R. similis*.
Thin, fresh, hand-cut sections were stained with Wiesner reagent to stain the lignin and observed under bright field transmitted light microscope. A) Lignified endodermis (arrow). B) Lignified inner cortical cells (black arrows) and endodermis (white arrow). C) & D) Extensive lignification of the endodermis (black arrows) and the sclerenchyma cell walls (white arrows). Magnification of images A & B: 400x; C & D: 100x.

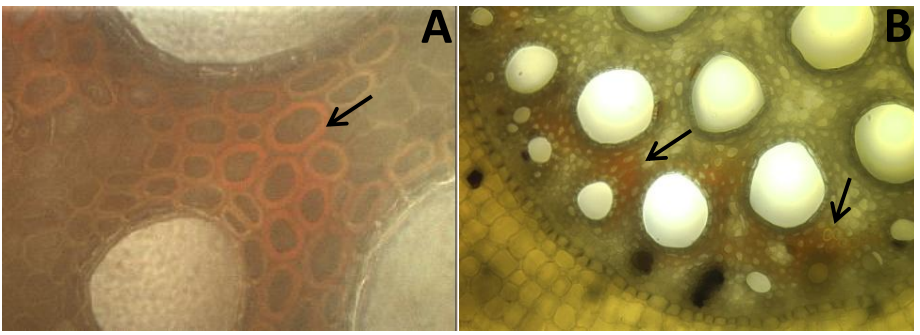


Figure 5.6: Tissue localisation of syringyl units of lignin in the root cross sections of *Radopholus similis*-resistant *Musa* genotype Long Tavoy at 6 weeks after inoculation with 1000 adults and juveniles of *R. similis*.
Thin, fresh, hand-cut sections were stained with Mäule reagent for syringyl lignin and observed under bright field transmitted light microscope. A) Secondary cell wall lignification of central vascular sclerenchyma cells (arrow) (x400) with syringyl lignin units. B) Lignification with syringyl units in the peripheral vascular sclerenchyma (arrows) (x100).

Cell wall lignifications in the *Musa* genotypes were confirmed by histochemical staining of the root cross sections with lignin specific dyes. When reacted with the Wiesner reagent, the lignified cells develop burgundy-red colour. With the Mäule reagent, the syringyl units of lignin develop bright or deep red colour.

Endodermis of all *Musa* genotypes became deep red coloured when treated with Wiesner reagent indicating the endodermal cell wall lignification especially after infection with *R. similis* (Figs 5.5C & D). Distinct pink colouration was observed in the endodermis of Yangambi km5 (Figs 5.5A & B). The reactions with Maule's reagents showed high syringyl units of lignin present in the central (Fig. 5.6A) and peripheral (Fig. 5.6B) vascular parenchyma (sclerenchyma) of *R. similis*-infected plants.

5.3.5. LIGNIN CONTENT OF ROOT CELL WALLS

The total lignin contents in the isolated roots cell walls of *R. similis*-infected and uninfected *Musa* genotypes are shown in the Figure 5.7. The results show that *R. similis* infection significantly ($P \leq 0.05$) increased the lignin content of Yangambi km5 roots at 6 weeks after inoculation. The root lignin content in the uninfected Yangambi km5 was lower than the root lignin content in uninfected Grande Naine at 6 weeks after inoculation (Fig. 5.7B). The post infection lignin content in Yangambi km5 was significantly ($P \leq 0.05$) higher than the post infection lignin content in Grande Naine at 6 weeks after inoculation.

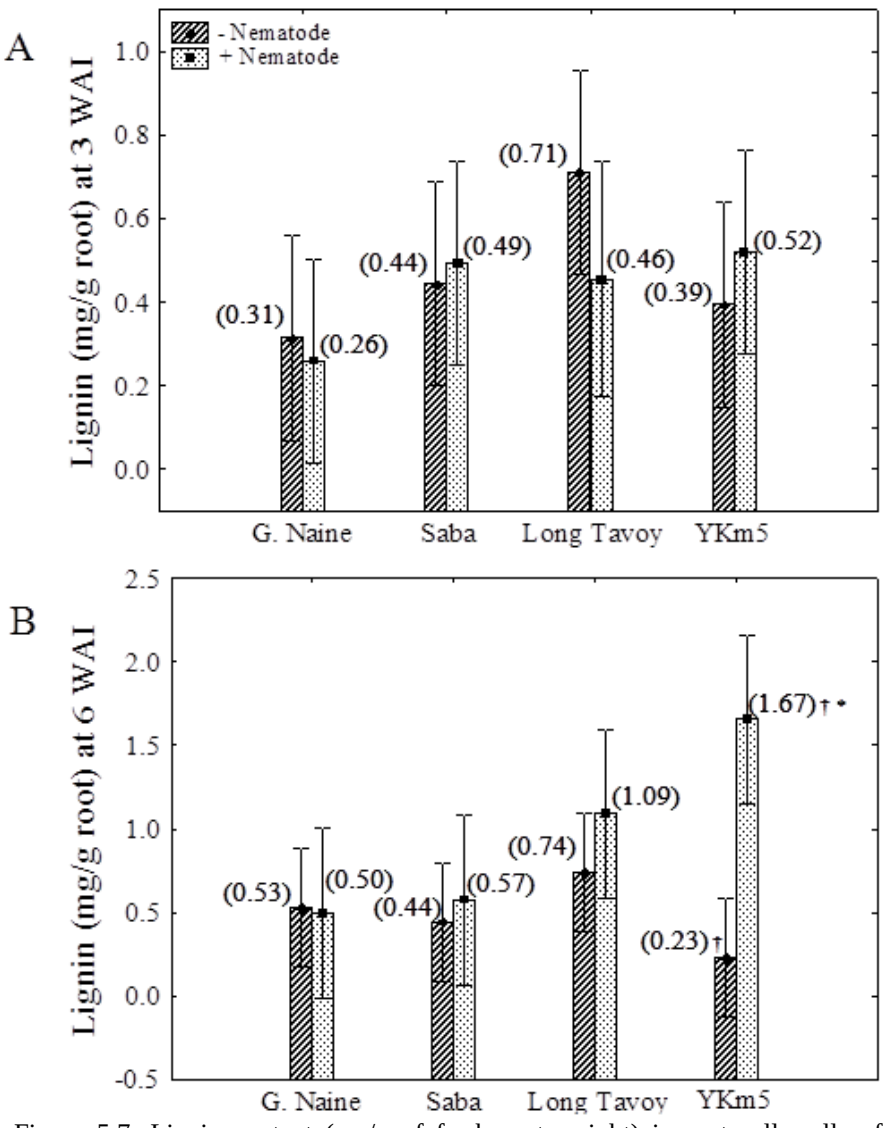


Figure 5.7: Lignin content (mg/g of fresh root weight) in root cell walls of *Radopholus similis*-infected and uninfected plants of resistant and susceptible *Musa* genotypes at 3 and 6 weeks after inoculation with 1,000 adults and juveniles of *R. similis*.

Mean lignin contents are presented between parentheses. Means followed by † indicate a significant difference ($P \leq 0.05$) between the *R. similis*-infected and uninfected plant of the same genotype. Means followed by * indicate a significant difference ($P \leq 0.05$) compared to the susceptible reference genotype Grande Naine. Error bars represent confidence intervals. (n=8).

G. Naine: Grande Naine; Ykm5: Yangambi km5.

There were no significant differences in lignin content among the *Musa* genotypes and between the *R. similis*-infected and uninfected plants of each genotype at 3 weeks after inoculation (Fig. 5.7A). In the histochemical staining, extensive lignification was observed constitutively in the older roots (6 weeks) compared to the younger (3 weeks) roots (Figs 5.3 & 5.4). This increase in the constitutive lignin content was observed only in Grande Naine and Long Tavoy during quantification (Fig. 5.7).

5.3.6. HISTOCHEMICAL STAINING OF *MUSA* ROOT SECTIONS FOR TOTAL PHENOLS

Observation of toluidine blue stained fresh root cross sections of *R. similis*-resistant and susceptible *Musa* genotypes illustrated the greenish blue stained lignified cell walls of vascular parenchyma (Fig. 5.8A). In the infected roots, the lesions were well stained with toluidine blue. The nematode infected necrotic cells were filled with phenolic substances (Fig. 5.8B).

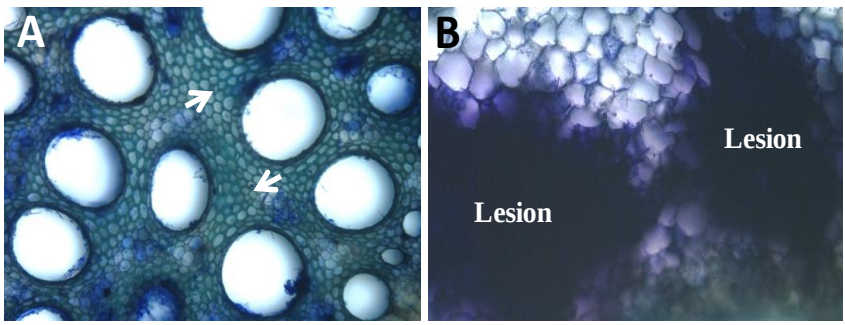


Figure 5.8: Tissue localisation of total phenols by toluidine blue staining in root cross sections of *Musa* genotypes at 6 weeks after inoculation with 1,000 adults and juveniles of *Radopholus similis*.
A) Blue-green staining of lignified vascular cell walls of uninfected Grande Naine root (x100). B) Phenols in the necrotic cells of *R. similis*-infected Grande Naine root (x100).

5.3.7. PHENOLIC CONTENT OF *MUSA* ROOTS

The total root phenol content was determined using the Folin-Ciocalteu method. Total phenol content was significantly ($P \leq 0.05$) lower in uninfected roots of all resistant *Musa* genotypes compared to the necrotic regions of *R. similis*-infected *Musa* roots at 3 and 6 weeks after inoculation. In contrast, in the susceptible genotype Grande Naine, the phenolic contents of uninfected and infected necrotic regions were comparable at 6 weeks after infection (Fig. 5.9B). The constitutive total phenol contents of all three resistant genotypes

were significantly ($P \leq 0.05$) lower than the constitutive phenol content in Grande Naine at 6 weeks after inoculation. No significant differences were observed between the total root phenol contents of the resistant and susceptible genotypes at 3 weeks after inoculation.

5.3.8. LOCALISATION OF FLAVONOIDS

Observations of DPBA treated root sections under an epifluorescence microscope with a FITC filter showed various fluorescing compounds. Green, bright yellow, yellow-gold, and orange fluorescence were observed in cells surrounding the necrosis in the cortex and aerenchyma of the root sections (Fig. 5.10). These fluorescent compounds are indicative of lignin and certain flavonoids according to the literature. After DPBA staining, lignin shows similar green or bright yellow fluorescence when observed under an epifluorescence microscope with FITC filter. Identification of the compounds based on the fluorescence is highly ambiguous. The fluorescing compounds were always observed in the cells and the intercellular spaces surrounding the necrotic lesions. They were never observed in the lesions.

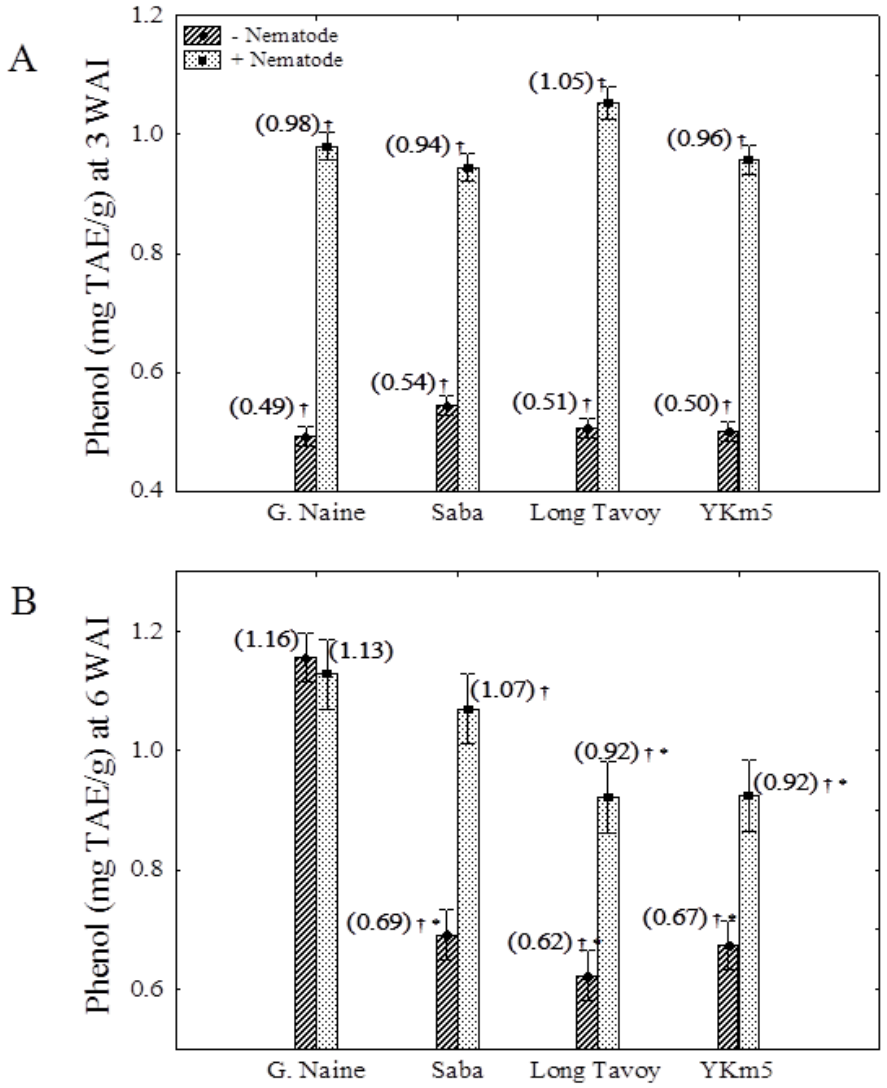


Figure 5.9: Total phenol contents (mg TAE/g roots) in the *Radopholus similis*-infected and uninfected roots of resistant and susceptible *Musa* genotypes at 3 and 6 weeks after inoculation with 1,000 adults and juveniles of *R. similis.*

Mean total phenol contents are presented between parentheses. Means followed by † indicate a significant differences ($P \leq 0.05$) between the *R. similis*-infected and uninfected plants of the same genotype. Means followed by * indicate a significant differences ($P \leq 0.05$) compared to the susceptible reference genotype Grande Naine. Error bars represent confidence intervals. G. Naine: Grande Naine; Ykm5: Yangambi km5.

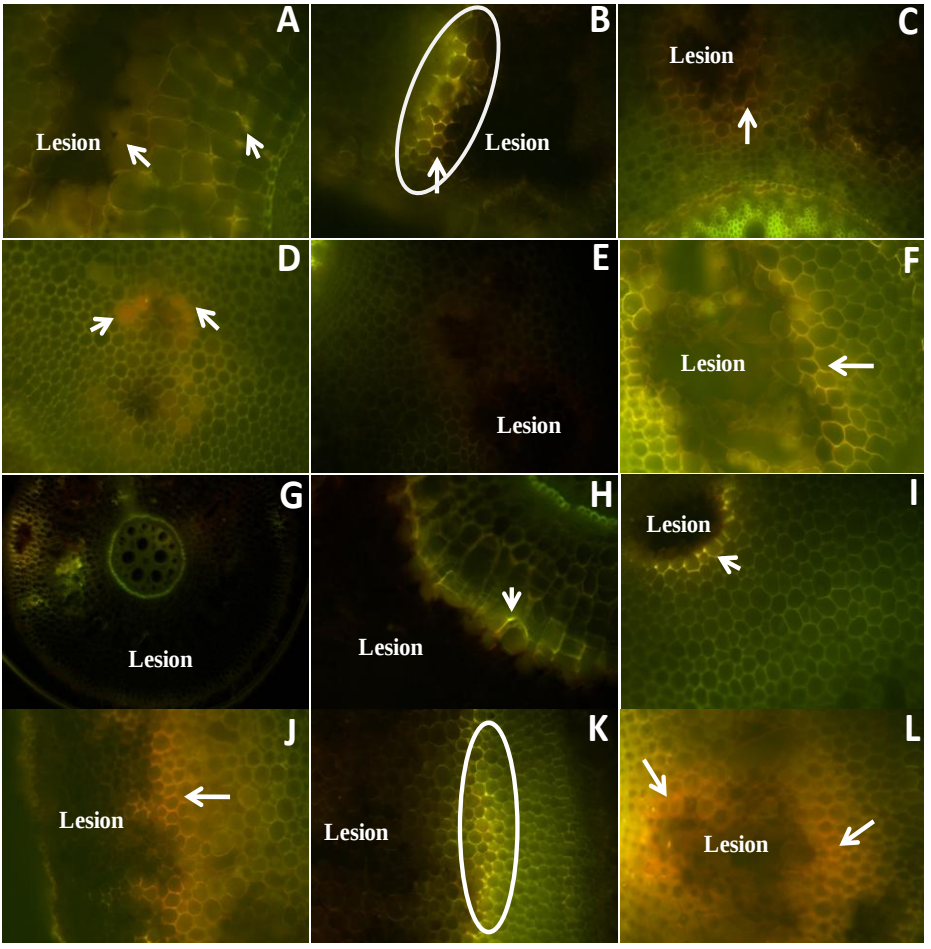


Figure 5.10: Fluorescence of phenolic compounds in root cross sections of *Radopholus similis*-infected susceptible and resistant *Musa* genotypes at 3 and 6 weeks after inoculation (WAI).

Thin, fresh, hand-cut sections were stained with DPBA and viewed under an epifluorescence microscope with a FITC filter. A) Bright and golden yellow fluorescing compounds in the intercellular spaces bordering the necrotic lesions in Grande Naine at 3 WAI. B) & C) Bright yellow and pale orange fluorescing compounds in the cell walls and intercellular spaces bordering the lesions in Grande Naine at 6 WAI. D) & E) Orange fluorescing compounds present in cells adjacent to lesions in Long Tavoy at 3 WAI. F) Bright and golden yellow compounds in the cell walls of Long Tavoy at 6 WAI. G) Green and yellow fluorescing cells next to a large lesion in Saba at 3 WAI. H) Bright yellow and green fluorescing compounds border the lesions in Saba at 6 WAI. I) & J) Bright, golden yellow and orange fluorescing compounds are bordering the lesions in Yangambi km5 at 3 WAI. K) & L) Green, yellow and orange fluorescing compounds present in the cell walls and intercellular spaces adjacent to lesions in Yangambi km5 at 6 WAI. Magnification of the images except G: 100x; G: 40x.

5.4. DISCUSSION

Lignin and phenols were tissue localised by histochemical analysis and quantitatively assessed in the *R. similis*-resistant and susceptible *Musa* roots to understand their involvement in the *R. similis*-resistance of *Musa* spp. The effect of *R. similis* infection on lignin and phenols accumulation was studied by comparing their constitutive levels in uninfected plants.

In our study, nematode infection was associated with a significant increase in the root weight of the resistant reference genotype Yangambi km5. Talwana *et al.* (2006) observed higher number of roots in the *R. similis*-infected banana genotypes Nabusa, Pisang Awak and Sukali Ndizi compared to the uninfected plants. This stimulation in root growth is a mechanism to compensate for the nematode damage by the dormant root primordia (Talwana *et al.*, 2006). Moreover, the nematode infection has increased the lignin content of Yangambi km5. The increase in root weight has been associated with an increase in the root secondary cell wall lignification (Fogain & Gowen, 1996). However, the increased root damage by nematodes results in reduced root system and loss of root weight and anchorage upon completion of the many nematode life cycles in the host roots.

No difference in the general structural root anatomy was found between the *R. similis*-resistant and susceptible *Musa* genotypes. The anatomical structures were similar to the previous descriptions of *Musa* roots (Acquarone, 1930; Wuyts, 2006). The nematode damage and necrosis were extensive in the outer cortex and aerenchyma. The necrosis has been extended to the endodermis and vascular bundle of a Yangambi km5 plant at early stage. Invasion of the vascular bundles by *R. similis* in *Musa* spp. has been occasionally reported (Mateille, 1994; Sarah *et al.*, 1996; Valette *et al.*, 1998). Poor lignification in the young roots might be the reason for this vascular invasion by the nematodes

Based on the histochemical staining, it is observed in our study that lignin was a major constituent of the cell walls of vascular bundles and endodermis. The cortex and aerenchyma cells were lignified to a very small extent. Similar observations were made previously (Fogain & Gowen, 1996; Wuyts *et al.*, 2007). This shows that the increased lignification is only a general defense response to protect the vascular bundle. Therefore, the damage to the plant can be reduced. Extensive lignification is not associated with the cortex cells that are directly involved in the defense to *R. similis*. Hence lignification is more likely to be associated with the plant's tolerance to *R. similis* preventing damage

to the plants and less likely to be associated with the resistance to nematode development, reproduction and multiplication.

Histochemical studies indicated a more extensive lignification of vascular bundles in older plants than in younger plants. Increased cell wall lignification with increase in age is documented in *Musa* spp. (Wuyts *et al*., 2007) and in other crops. For instance, the proximal roots of wheat contain more lignin than the distal younger roots (Rengel *et al*., 1994). The higher levels of indole acetic acid (IAA) in younger root tissues inhibit phenylalanine ammonia lyase activity and lignin deposition (Burnell, 1988). However, in our study the quantitative assessment of lignin content based on the thioglycolic acid assay did not confirm extensive lignification of older roots of all genotypes.

Histochemical staining showed more intense secondary cell wall lignifications in the endodermis, xylem-accompanying cells and the vascular parenchyma of *R. similis*-infected plants compared to the uninfected plants of all *Musa* genotypes. Stronger lignification in the endodermis and vascular bundle is considered as a general defence response to nematode infection creating a physical and chemical barrier to the vascular invasion (Keen, 1992; Zacheo *et al*., 1997; Valette *et al*., 1998; Wuyts *et al*., 2007).

Varying levels of lignin contents were observed in the infected and uninfected plants at 3 weeks after infection. However, the infection of *R. similis* increased the cell wall lignin content of Yangambi km5 plants at 6 weeks after infection. The post-infection increase in the lignin content of Yangambi km5 was 7 times higher than in the uninfected plants. In Grande Naine, the extended cellular damage due to nematode infection might have caused a reduction in total lignin content compared to the uninfected plants. A prominent increase in lignin contents due to *R. similis*-infection was reported in *R. similis*-resistant *Musa* genotypes Pisang Jari Buaya and Calcutta 4 as well as in the susceptible genotype Grande Naine. However, a reduction in lignin content was in Yangambi km5 after *R. similis* infection (Wuyts *et al*., 2007).

Histochemical localisation of total phenols by staining with toluidine blue showed that the phenolic substances are major constituents of the nematode infected necrotic cells. Toluidine blue staining showed the lignified cell walls of vascular bundles in the roots. No preformed phenolic cells were observed in the *R. similis*-resistant and susceptible *Musa* genotypes as previously reported in the *R. similis*-resistant *Musa* genotypes Yangambi km5 and Gros Michel (Fogain & Gowen 1996).

The results of the quantitative assay of total phenols by the Folin-Ciocalteu assay corroborate the histochemical localisation. The nematode infection has almost doubled the total phenols contents in all *Musa* genotypes at 3 weeks after infection. This drastic increase in total phenols was observed in the *R. similis*-resistant *Musa* genotypes at 6 weeks after infection. Infected regions were sampled for this phenolic assay. Hence the increase in total phenolic contents is due to the phenols accumulation in the lesions. The enhanced synthesis of phenols could be due to the biosynthesis or accumulation of phytoalexins in the nematode feeding cells with anti-nematode properties. However, the Folin-Ciocalteu assay is a very general test for phenols. Further studies using analytical techniques such as chromatography, NMR spectroscopy and mass spectrometry can facilitate the structural identification of the individual compounds. In our further study we focused on identifying the secondary metabolites accumulated or biosynthesised in the necrotic lesions. The high constitutive phenols content in Grande Naine is probably due to high polyphenolic content in Grande Naine or increased polyphenol content due to tissue damage during sampling and handling. The older *in vitro* Grande Naine roots in tissue cultures also show intense browning and secrete dark brown compounds in the medium without any infection or physical injury.

Histochemical staining of *R. similis*-infected roots with Neu's reagent showed the presence of fluorescing compounds bordering the lesions in all the *Musa* genotypes. These fluorescing compounds could be lignin accumulation induced by the nematode infection to protect the adjacent cells from nematode invasion. These fluorescing compounds were also reported as candidates of certain flavonoids (Valette *et al*., 1998; Wuyts *et al*., 2007). However, it is highly ambiguous to identify the secondary metabolites based on the fluorescence. These compounds were mainly present in the cell walls and intercellular spaces suggesting their role as defence related signalling molecules. Phenolic compounds were suggested to play a key role in internal signalling in plants (Ferrer *et al*., 2008). No striking differences were observed between the susceptible and resistant genotypes in the presence of constitutively fluorescing compounds as previously reported (Valette *et al*., 1998). The phenols in the lesions were non-fluorescent. Our further study (Chapter 6.1) reports the results using analytical techniques for the unambiguous identification of the secondary metabolites in the nematode infected root tissues.

5.5. CONCLUSION

Radopholus similis infection has increased the root secondary wall lignification of the endodermis and vascular bundles of resistant and susceptible *Musa* genotypes. This increased lignification is a general defense response to protect the vascular bundle to reduce damage to the plant. Extensive lignification is not associated with the cortex cells that are directly involved in the defense with nematodes. Hence lignification is more likely to be associated with the plant's tolerance to *R. similis* damage than with resistance to nematode development, reproduction and multiplication.

Preformed phenolic cells were not found to be related with nematode resistance in the studied *Musa* genotypes. The nematode infection has dramatically increased the total phenol contents of resistant plants. Phenolic secondary metabolites are major constituents of the nematode infected necrotic cells. Detailed analytical studies are essential to identify the secondary metabolites that are induced in nematode infection sites. Further studies are necessary for the structural identification of these secondary metabolites involved in the necrotic lesions.

CHAPTER 6

PHENYLPHENALENONE-TYPE PHYTOALEXINS INVOLVED IN THE PLANT RESISTANCE TO PARASITIC NEMATODES

RATIONALE AND OUTLINE

"Phytoalexins are low molecular weight antibiotic compounds, which are synthesized and accumulated in plants after exposure to pathogens or stress" (Paxton, 1980). Biosynthesis of phytoalexins is well accepted as a plant's defense strategy to fight biotic stress (Bednarek & Osbourn, 2009). However, the real contribution of phytoalexins to plant's resistance to nematodes remains elusive as only few secondary metabolites were isolated and identified from plants and examined for nematicidal activity (Chitwood, 2002; Wuyts, 2006). Moreover, the majority of the studies were based on histochemical staining lacking analytical studies for proper identification (Wuyts *et al*., 2007; Svatoš, 2010).

Phenylphenalenones are phenolic secondary metabolites originally identified as pigments in plants belonging to the monocot family Haemodoraceae (Cooke & Edwards, 1981; Dora *et al*., 1993; Hölscher & Schneider, 1997; Opitz & Schneider, 2002). These compounds show an organ-specific distribution such as in apical meristems, root caps, cortex and epidermis (Opitz *et al*., 2003) and in the secretary cavities of leaves and flowers (Hölscher & Schneider, 2007) of Haemodoraceae. Phenylphenalenones are found to act as phytoalexins, elicited by pathogenic infections in plants belonging to the Musaceae family (Luis *et al*., 1993, 1995). In *Musa* spp., phenylphenalenones are elicited in the roots and rhizomes by the fungus *Fusarium oxysporum* f. sp. *cubense* causing Panama disease (Luis *et al.*, 1994; 1996) and by the burrowing nematode *Radopholus similis* (Cobb, 1893) Thorne, 1949 (Binks *et al*., 1997), in the aerial parts by the fungus *Mycosphaerella fijiensis* causing Black Sigatoka leaf streak disease (Luis *et al*., 1993, 1994; Otálvaro *et al*., 2007) and in the fruits by the fungus *Colletotrichum musae* causing anthracnose (Kamo *et al*., 1998, 2001). Report on phenylphenalenones elicitation in response to nematode infection is limited to the isolation and identification (Binks *et al*., 1997). Systematic studies involving tissue localisation, isolation and identification of the compounds in resistant and susceptible cultivars, followed by examination of the anti-nematode properties of the compounds have not been performed so far.

Molecular level information on the host-nematode interactions of migratory endoparasitic nematodes is only emerging recently and scarce compared to the wealth of information available for sedentary endoparasitic nematodes (Haegeman *et al*., 2010). Some studies are dedicated to the investigation of phytochemicals in *R. similis*-resistant and susceptible *Musa* cultivars (Fogain & Gowen, 1996; Valette *et al*., 1998; Collingborn *et al*., 2000;

Wuyts *et al*., 2007). The majority of these studies used non-specific tests for phenols and histochemical staining suggesting the involvement of phenylpropanoids in *Musa*-*R. similis* interactions (Collingborn *et al*., 2000). In our study, many analytical techniques such as liquid chromatography, mass spectrometry, mass spectrometry imaging and nuclear magnetic resonance spectroscopy were combined to precisely identify the phytoalexins involved in the *Musa*-*R. similis* interactions. The anti-*R. similis* properties of the isolated compounds were also evaluated. The objective of our study was to identify and quantify the phytoalexins followed by assessment of the anti-nematode properties of the phytoalexins elicited in the *R. similis*-resistant and susceptible *Musa* cultivars. It was also our objective to develop a strategy that could be applicable to study other host-nematode interactions by combining an array of technologies.

Our results are reported in this chapter in two sections. The first section presents the results of tissue localisation and profiling of phenylphenalenones in a *R. similis*-resistant and a susceptible *Musa* genotypes. The second section presents the results of the *in vitro* bio-assays evaluating the anti-*R. similis* properties of the phenylphenalenones.

CHAPTER 6.1: CELL-SPECIFIC LOCALISATION AND PHYTOCHEMICAL PROFILING OF PHENYLPHENALENONE-TYPE PHYTOALEXINS IN *RADOPHOLUS SIMILIS*-RESISTANT AND -SUSCEPTIBLE *MUSA* ROOTS

6.1.1. INTRODUCTION

Plants use a wide range of phytochemicals to defend themselves from invading pathogens as they cannot move to escape from the pathogens (Field *et al*., 2006). The interaction between a host plant and parasitic nematodes affects the molecular and physiological pathways in host plants (Grunewald *et al*., 2009). It also induces many host responses including metabolite expressions such as production of phytoalexins (Gheysen & Fenoll, 2002). Little information is available on the changes in plant metabolites due to interactions with migratory endoparasitic nematodes. The *Musa* cultivar Yangambi km5 has been identified and used as a reference cultivar, resistant to *R. similis* (Speijer & De Waele, 1997; Fogain & Gowen, 1998). This cultivar is also resistant to Black Sigatoka leaf streak disease (Fouré *et al.,* 1990; Otálvaro *et al*., 2007). Based on the results of our previous experiments (Chapters 3 & 4), it is clear that in resistant *Musa* cultivars the post-infectional development and reproduction of *R. similis* is inhibited. However, the mechanism of this nematode resistance is elusive.

Phenylphenalenones were reported as phytoalexins produced in response to *R. similis* infection in *Musa* roots (Binks *et al*., 1997). A recent study (Otálvaro *et al*., 2007) suggested the involvement of phenylphenalenones in the resistance of Yangambi km5 to the Black Sigatoka leaf streak disease. It is anticipated that phenylphenalenone-type phytoalexins might also be involved in the resistance of Yangambi km5 to *R. similis*. Investigations on phenylphenalenone profiles of *R. similis*-infected and uninfected plants of Yangambi km5 and Grande Naine, a reference *Musa* cultivar susceptible to *R. similis*, form the main focus of this part of the present chapter.

The matrix-free laser desorption/ionisation mass spectrometric imaging (LDI-MSI) technique was successfully used in *Hypericum* sp. and the model plant *Arabidopsis thaliana* for cell-specific localisation of UV-absorbing-secondary metabolites (Hölscher *et al*., 2009). In our study, LDI-MSI is used on *Musa* roots to specifically map the secondary metabolites elicited in the root cells after nematode infection. The knowledge on the cellular localisation will deepen the understanding on the function of the secondary metabolites. LDI-

MSI provides information on spatial distribution of the secondary metabolites and the *m/z* (mass-to-charge ratio) values of the compounds. The secondary metabolites corresponding to the mass were identified and confirmed by a multidimensional approach, combining the analytical techniques such as high performance liquid chromatography (HPLC), proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and ultra performance liquid chromatography coupled with mass spectrometry (UPLC-MS). NMR techniques are less sensitive and amplification techniques such as commonly used for DNA and RNA are not available for metabolites (Hölscher *et al*., 2009). However, when sufficient amounts of the metabolites are available, NMR analysis is the most powerful tool to obtain structural information of secondary metabolites. The UPLC-MS technique provides information about mass of a metabolic signal and the modern day MS techniques are highly sensitive. The combination of analytical techniques namely LDI-MSI, HPLC, ^{1}H NMR and UPLC-MS were used to localise and identify the secondary metabolites involved in the *Musa*-*R. similis* interactions.

6.1.2. MATERIALS AND METHODS

6.1.2.1. Plants

The *R. similis*-susceptible cultivar Grande Naine (*Musa acuminata* AAA, Cavendish subgroup, ITC 1256) and the *R. similis*-resistant cultivar Yangambi km5 (*Musa acuminata* AAA, Ibota subgroup, ITC 1123) were initially obtained from the *Musa* germplasm collection maintained at the International Transit Centre (ITC), K.U.Leuven, Belgium. The plant material was proliferated, regenerated and rooted in test tubes as explained in Chapter 2 (2.2.2). The plantlets were grown in growth chambers at 28 °C and 16 h photoperiod. Eight-weeks-old rooted tissue culture plantlets were planted in 8 l pots filled with sand and potting soil (2:1). The plants were maintained under greenhouse conditions and fertilized at 10 days intervals throughout the experiment. In the greenhouse, the ambient day and night temperatures were 27 and 20 ºC, respectively, relative humidity was 80% and the photoperiod was 12 h.

6.1.2.2. Nematodes

A population of *R. similis* originally isolated from banana roots in Uganda was used in the experiment. This population was maintained and multiplied monoxenically on sterile carrot discs at 25±1 °C in the dark (Speijer & De Waele, 1997). The population from Uganda was characterized by a high

reproductive fitness (Fallas *et al*., 1995). To obtain the inoculum, adults and juveniles of *R. similis* were extracted from the carrot discs by the maceration-sieving technique (Speijer & De Waele, 1997). The volume of the nematode suspension was adjusted to a concentration of 1,000 active adult and juveniles of *R. similis* in 2 ml of water.

Figure 6.1: *Musa* plants arranged in a randomized block design in the greenhouse to study the phytoalexins synthesis in response to *Radopholus similis* infection.

6.1.2.3. Experimental set-up

The experiment consisted of 12 plants of each cultivar. At 8 weeks after planting, six plants of each cultivar were infected with 8,000 living vermiform nematodes by pipetting 16 ml of the nematode suspension into inoculation holes made in the soil near the plant root zone. Six other plants of each cultivar were included as control plants uninfected with nematodes. The plants were placed in the greenhouse according to a randomized block design.

6.1.2.4. Sampling

All the plants were uprooted at 12 weeks after inoculation. The 12 weeks interval was allowed to obtain a higher concentration of the phytochemicals to carry out all analyses and to perform the bio-assays. The root systems were carefully washed free of soil (without destroying the root epidermis) under running tap water. Roots were blot-dried, fresh root and shoot weights were measured, the root systems were snap-frozen in liquid nitrogen and stored at -80

°C for phytochemical analysis. A small portion of young roots with newly developing necrotic lesions were preserved separately for localisation of phenylphenalenones using LDI-MSI. The scheme of the analyses performed on the roots is illustrated in Figure 6.2.

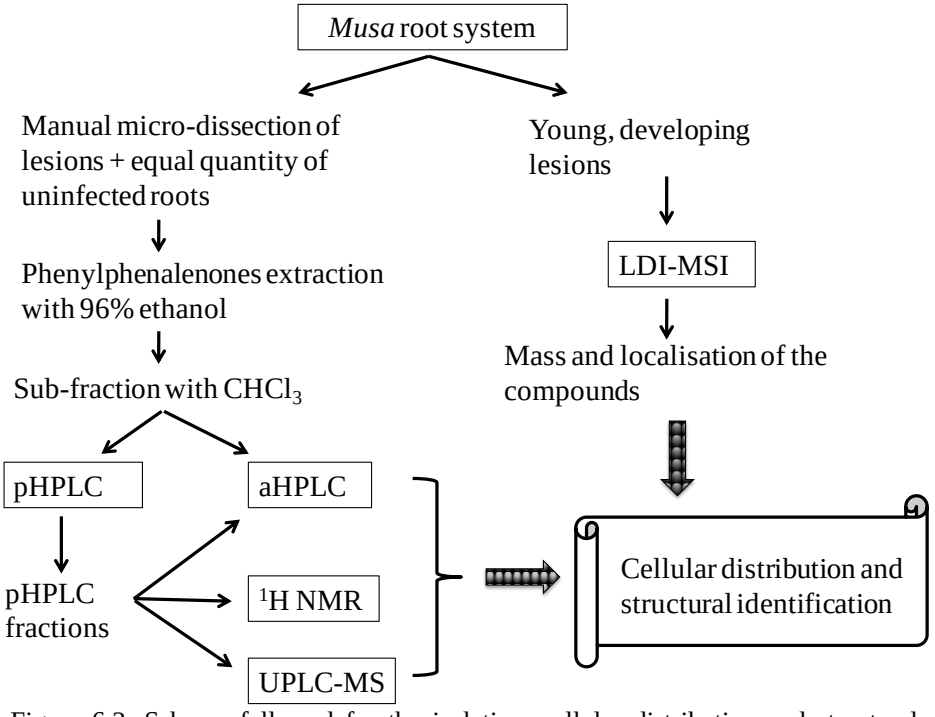


Figure 6.2: Scheme followed for the isolation, cellular distribution and structural identification of phenylphenalenones.
$CHCl_3$: Chloroform; pHPLC: preparative high performance liquid chromatography; aHPLC: analytical high performance liquid chromatography; ^{1}H NMR: proton nuclear magnetic resonance spectroscopy; UPLC-MS: ultra performance liquid chromatography-mass spectrometry; LDI-MSI: Matrix-free laser desorption/ionization mass spectrometry imaging.

The ^{1}H NMR and the UPLC-MS analyses were performed in co-operation with the NMR and MS groups of the Max-Planck-Institute for Chemical Ecology in Jena, Germany. The LDI-MSI work was performed in co-operation with the Laboratory of Organic Chemistry and Macromolecular Chemistry of the Friedrich Schiller University in Jena, Germany.

6.1.2.5. Extraction and analysis of phenylphanalenones

From the nematode-infected plants of Yangambi km5 and Grande Naine, necrotic root tissues were manually micro-dissected and weighed. The same amounts of healthy roots were sampled from the uninfected plants of each cultivar as control. Samples were ground in liquid nitrogen. This was followed by an immediate extraction using 96% ethanol. The extract was filtered by using filter paper. This crude extract was evaporated using a rotary evaporator (Rotavapor R-114, waterbath B-480, Büchi Labortechnik AG, Flawil, Switzerland; Pump system: Vaccubrand Diaphragm Vaccum Pump type MZ 2C, Vaccubrand, Wertheim, Germany) to remove the solvent. The resultant was sub-fractioned between chloroform/H_2O, followed by partition between ethyl acetate/H_2O and *n*-butanol/H_2O using a separating funnel. All three sub-fractions obtained from each sample were collected separately, filtered and evaporated to remove the solvents and stored in small vials at room temperature.

6.1.2.6. Thin layer chromatography (TLC) analysis

TLC was used to obtain a preliminary idea about the compounds in the sub-fractions prior to using other analytical methods. The chloroform sub-fraction from Grande Naine was developed in the TLC system along with the chloroform sub-fraction of *Strelitzia reginae* roots as a standard for comparison (Hölscher & Schneider, 2000). About 15 µl of the samples were loaded on the TLC sheets (TLC aluminium sheets, 20 x 20 cm, silica gel 60 F_{254}, Merck, Darmstadt, Germany). The TLC developing system consisted of 3:2 toluene and acetone. The retention factors (R_f) of the separated compounds from Grande Naine were compared with the R_f of known compounds from *S. reginae* for a tentative identification.

6.1.2.7. Preparative HPLC (pHPLC)

Purifications of the sub-fractions were done on a Merck-Hitachi LiChrograph preparative chromatography system with an L-6200A gradient pump, L-4250 UV-VIS detector (Merck-Hitachi, Darmstadt, Germany). The preparative HPLC runs were performed on a reverse phase RP18 column (LiChrospher® 100 RP18; 250 x 10 mm, 10 µm) at a constant flow rate of 3.5 ml/min using a binary solvent system: solvent A, water with 0.1% trifluoroacetic acid (TFA) and solvent B, acetonitrile. The HPLC gradient system was started with 15% B and linearly increased to 95% B at 120 min and

held for 5 min, then brought back to the 15% B initial condition at 135 min and held for 5 min for the column re-equilibration for the next injection. Wavelength studied at 284 nm. All the peaks individually collected and purified using pHPLC were subjected to analytical HPLC, ^{1}H NMR and UPLC-MS to detect phenylphenalenones.

6.1.2.8. Analytical HPLC (aHPLC)

Tentative identification and purification of phenylphenalenones from the *Musa* root extracts were carried out by analyzing the sub-fractions using an analytical HPLC system (Agilent series HP1100; binary pump G1312A; auto-sampler G1313A; diode array detector (DAD) G1315B). The aHPLC was performed using a reverse phase analytical column, LiChrospher® 100 RP18 (Merck, Darmstadt, Germany). Each sub-fraction was dissolved in methanol mixture at a concentration of 10 mg/ml. In each run, 25 µl of the sub-fraction was separated at room temperature at a constant flow rate of 0.8 ml/min using a binary solvent system: solvent A, water with 0.1% TFA and solvent B, acetonitrile. The chloroform sub-fractions were separated using the aHPLC gradient started with 20% of B and linearly increased to 50% B at 60 min, 95% B at 65 min and back to 20% B initial condition at 70 min for the column re-equilibration for the next injection. The diode array detector (Agilent diode array detector (DAD) G1315B) was used to record the online spectra at 254, 270, 284, 360 and 450 nm wavelengths.

6.1.2.9. ^{1}H NMR spectroscopy

^{1}H NMR spectra were recorded on a Bruker Avance DRX 500 NMR spectrometer (Bruker, Karlsruhe, Germany) in acetone-d_6 at 24 °C. The resonance frequency was 125.13 MHz for ^{1}H. Chemical shifts (δ) are given relative to tetramethylsilane (TMS), which was used as an internal standard for referencing ^{1}H NMR spectra. The spectrometer was equipped with a broad-band probe (5 mm, z-gradient, automatic tuning and matching). XwinNMR 3.1 software (Bruker-Biospin) was used to control the spectrometer and data processing.

6.1.2.10. UPLC-MS analysis

The LC-MS system consisted of an Ultimate 3000 series RSLC (Dionex, Sunnyvale, California) system and orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). HPLC was performed using Acclaim C18

Column, (150 x 2.1 mm, 2.2 µm, Dionex) at a constant flow rate of 300 µl/min using a binary solvent system: solvent A, water with 0.1% formic acid and solvent B, acetonitrile with 0.1% formic acid. The HPLC gradient system started with 5% B, linearly increased to 98% B at 28 min and held for 3 min, then brought back to the 5% B initial condition and held for 5 min for the column re-equilibration for the next injection. Full scan mass spectra were generated using 30,000 resolving power.

6.1.2.11. Fixation of plant material for LDI-MSI

Initially, the cryo-sectioning and fixation performed at -25 °C produced root cross sections of 60 µm thickness (Cryostat CM 1850, Leica Microsystems, Nußloch, Germany). However, the fixative used in cryo-sectioning obstructed the LDI-MSI procedure. Alternatively, thin, small longitudinal sections of root epidermis at the region of nematode infection were performed using a razor blade. The root sections were fixed on a carbon conductive adhesive tape (Plano, Wetzlar, Germany). The sample on the conductive tape was fixed on the conductive, indium tin oxide (ITO) coated glass slides (Bruker Daltonic, Bremen, Germany). A gel-liner (Staedtler triplus gel-liner, silver, 0.4 mm; Staedtler-Mars, Nürnberg, Germany) was used to place marks surrounding the sections to define their position. The gel marked points were used as reference points or guidelines to teach the LDI-MSI system to position the regions of root samples to be analyzed.

6.1.2.12. LDI-MSI on the ultraflex III® mass spectrometer

An Ultraflex III® 421 (Bruker Daltonics, Bremen, Germany) was used for the analysis. The instrument was equipped with a Nd:YAG laser with an emitting wavelength of 355 nm. All spectra were measured in the negative reflectron mode. For measuring the pixels, the minimum laser focus setting was used (corresponding to a diameter of about 10 µm laser, under usage of oversampling for the measurement with a raster of 20 x 20 µm). For each raster point, a spectrum was accumulated with 100 laser shots, a shot rate of 40 Hz and fixed laser intensity. For image reconstruction, the FlexImaging v.2.0 software (Bruker Daltonics, Bremen, Germany) was used. Within all prepared mass images, the signal intensity is represented by the corresponding color intensity for the represented mass.

6.1.3. RESULTS

6.1.3.1. Root damage and root lesions

The root damage caused by *R. similis* infection was severe in the susceptible *Musa* cultivar Grande Naine compared to the resistant *Musa* cultivar Yangambi km5 (Figs 6.3 & 6.4).

Visual observation of *R. similis*-infected roots of Yangambi km5 showed that the young developing as well as the old lesions were small, discontinuous and dark coloured and non-expanding as in hypersensitive reactions (Figs 6.4E-G). The lesions were large, continuous and tunnel-like in Grande Naine (Figs 6.4A & B). Moreover, the root damage was extended to the root bases in the Grande Naine corms (Fig. 6.4C). No corm infection was observed in Yangambi km5.

6.1.3.2. Sub-fraction weights

The mean weights of each sub-fraction isolated from the root extracts of Yangambi km5 and Grand Naine are listed in Table 6.1. Higher amounts of all the sub-fractions were isolated from the nematode-infected plants of both cultivars. The chloroform sub-fraction extracted from the nematode-infected Yangambi km5 roots weighed about 2.7 times of the weight of chloroform sub-fraction of uninfected plants. In Grande Naine, the chloroform sub-fraction of nematodes infected roots weighed 1.6 times of the weight of the chloroform sub-fraction from uninfected plants. A striking difference was observed in the colour intensity between the ethanol extracts of *R. similis*-infected and uninfected plants (Fig. 6.4D). The chloroform sub-fractions of both cultivars showed signals of candidate phenylphenalenones in TLC and HPLC analyses.

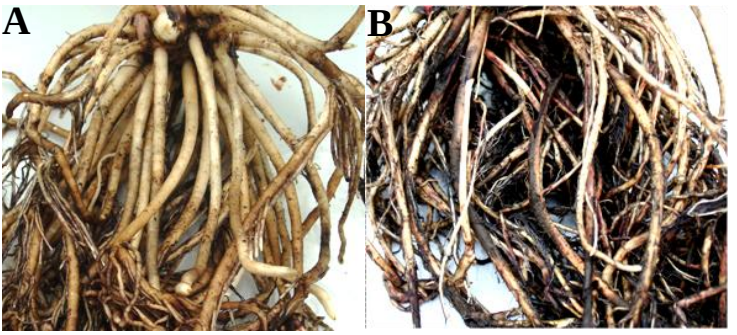


Figure 6.3: The root system of *Musa* cultivar Grande Naine. A) Uninfected healthy roots. B) *Radopholus similis*-infected roots at 12 weeks after infection.

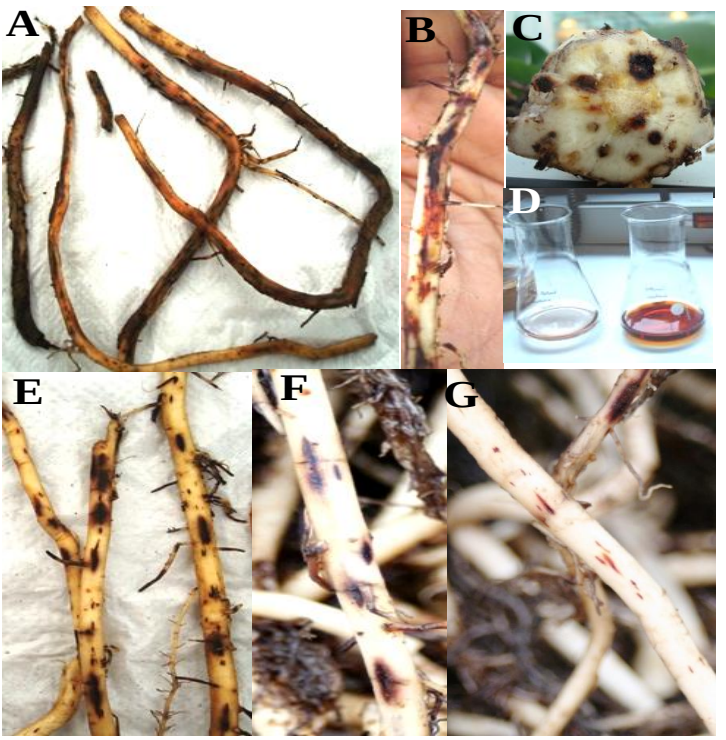


Figure 6.4: Root necrosis caused by *Radopholus similis* infection in susceptible (Grande Naine) and resistant (Yangambi km5) *Musa* cultivars. A) Well developed, tunnel-like, dark brown, older lesions on Grande Naine. B) Young, developing, reddish brown lesions on Grande Naine. C) Lesions on the root bases of a Grande Naine corm. D) The ethanol extracts of uninfected (left) and *R. similis*-infected (right) Grande Naine roots. E) & F) Small, discontinuous, hypersensitive-like, dark brown, older lesions on Yangambi km5. G) Small, young, developing reddish brown to dark brown lesions on Yangambi km5.

Table 6.1. Weights of the sub-fractions isolated from the *Musa* root extracts.

Details	Weight of the sub-fractions (mg/g of roots)		
	Chloroform	Ethyl acetate	*n*-Butanol
Grande Naine^{S}			
Infected region	1.97	0.85	2.58
Uninfected plant	1.25	0.30	2.08
Yangambi km5^{R}			
Infected region	4.67	1.65	2.55
Uninfected plant	1.74	0.31	2.42

^{S} Susceptible *Musa* cultivar; ^{R} Resistant *Musa* cultivar; Infected: *Radopholus similis*-infected.

6.1.3.3. Thin layer chromatography (TLC)

The chloroform sub-fraction separated from the ethanol extracts of the *R. similis*-infected Grande Naine roots were analysed by TLC for the occurrence of phenylphenalenones. A chloroform sub-fraction isolated from *S. reginae* plants was used as a standard to compare the retention factor (R_f) of the known phenylphenalenones. The known phenylphenalenones anigorufone and hydroxyanigorufone were tentatively identified in the chloroform sub-fraction of Grande Naine (Fig. 6.5).

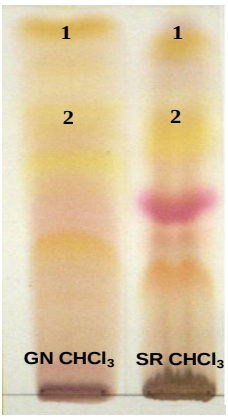


Figure 6.5: TLC chromatogram of chloroform subfractions.
GN $CHCl_3$: Grande Naine chloroform sub-fraction; SR $CHCl_3$: *S. reginae* chloroform sub-fraction. **1**: anigorufone and **2**: hydroxyanigorufone.

6.1.3.4. Purification of phenylphenalenones by HPLC

The chloroform sub-fractions of *R. similis*-infected and uninfected root extracts of Yangambi km5 and Grande Naine were purified by pHPLC. Nineteen fractions from Yangambi km5 and 16 fractions from Grande Naine were eluted from the chloroform sub-fractions of *R. similis*-infected plants during the pHPLC runs.

All these fractions were individually collected and subjected to aHPLC, ^{1}H NMR and UPLC-MS analyses for identification. Nine phenylphenalenones-type phytoalexins were structurally identified based on ^{1}H NMR analyses. The structures of the identified compounds are illustrated in Figure 6.6. The compounds will be numbered throughout the chapter as in Figure 6.6 for the ease of identification.

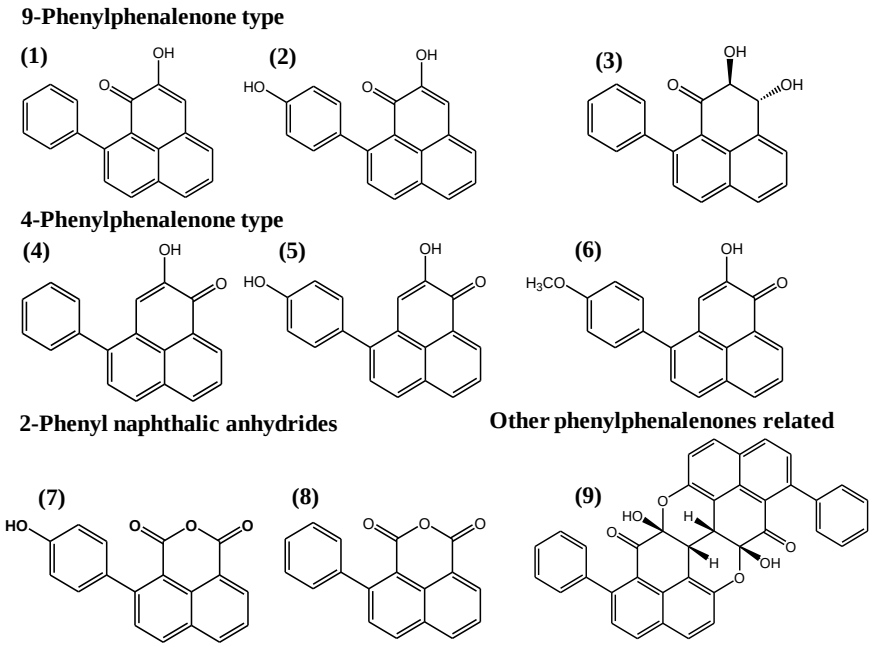


Figure 6.6: Structure of all the isolated phenylphenalenone-type phytoalexins. (**1**) anigorufone; (**2**) hydroxyanigorufone; (**3**) (2*S*,3*R*)-2,3-dihydro-2,3-dihydroxy-9-phenylphenalen-1-one; (**4**) isoanigorufone; (**5**) irenolone; (**6**) methlyirenolone; (**7**) 4-(4-hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromene-1,3-dione; (**8**) 4-phenyl-1*H*,3*H*-benzo[*de*]isochromene-1,3-dione; (**9**) anigorootin. The numbers between the parentheses corresponding to each compound will be used for identification throughout the chapter.

The identified peaks are marked with the corresponding compound numbers in the aHPLC chromatograms (Fig. 6.7B & D). The ^{1}H NMR analysis of other peaks of the aHPLC run did not clearly identify further metabolites but mainly indicated mixtures or suggested few unknown natural products. The chromatograms of the uninfected plants were comparable to the solvent (methanol) run (Figs 6.7A & C). The aHPLC results clearly exhibited the differences between the nematode-infected regions and uninfected plants. Many *R. similis*-infection-induced signals were observed in the chromatograms of the chloroform sub-fractions (Figs 6.7B & D).

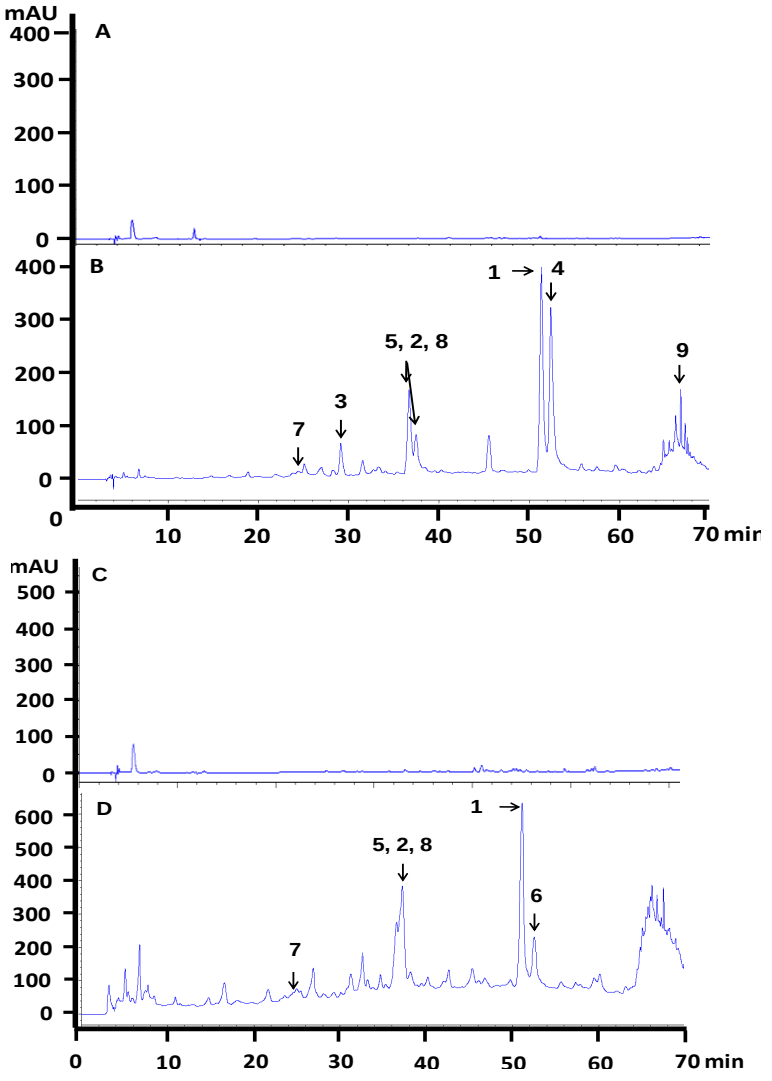


Figure 6.7: Analytical HPLC chromatograms of chloroform sub-fractions separated from the ethanol extracts of *Musa* spp. A) & B) Yangambi km5. C) & D) Grande Naine. A) Chromatogram from the uninfected roots. B) Chromatogram of *Radopholus similis*-infected root necrotic lesions. C) Chromatogram from the uninfected roots. D) Chromatogram of *R. similis*-infected root necrotic lesions. Each peak is numbered with the corresponding compound number as in Figure 6.6.

The identified compounds and their occurrence in Yangambi km5 and Grande Naine are listed corresponding to their aHPLC retention time (Table 6.2).

Table 6.2. The occurrence of identified phenylphenalenones in the resistant and susceptible *Musa* cultivars based on HPLC and ^{1}H NMR analyses.

R_t (min)	Name of the compound	Ykm5	GN
23.35	4-(4-Hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromene-1,3-dione (**7**)	X	X
29.19	(2*S*,3*R*)-2,3-dihydro-2,3-dihydroxy-9-phenylphenalen-1-one (**3**)	X	-
36.87	Irenolone (**5**)	X	X
37.59	Hydroxyanigorufone (**2**)	X	X
37.59	4-Phenyl-1*H*,3*H*-benzo[*de*]isochromene-1,3-dione (**8**)	X	X
51.65	Anigorufone (**1**)	X	X
52.67	Isoanigorufone (**4**)	X	-
52.93	Methylirenolone (**6**)	-	X
66.75	Anigorootin (**9**)	X	-

The R_t listed in the table is based on the aHPLC analysis. R_t: retention time; Ykm5: Yangambi Km5; GN: Grande Naine; X: present; -: absent.

6.1.3.5. Structural identification

Nine secondary metabolites were isolated and identified from the *R. similis*-infected *Musa* plants. All of them were identified as phenylphenalenone-type compounds based on ^{1}H NMR spectroscopy. The ^{1}H NMR spectra of all these compounds showed proton signals of phenylphenalenones-type characteristics. The compounds were unambiguously identified by comparing the chemical shifts (δ, ppm) and spin-spin couplings of the aromatic, aliphatic and methoxy signals from the ^{1}H NMR spectrum of each fraction (*e.g.* Fig. 6.8B) with the ^{1}H NMR spectrum of authentic phenylphenalenones previously reported from *Musa* spp. and other plant sources (*e.g.* Fig. 6.8A).

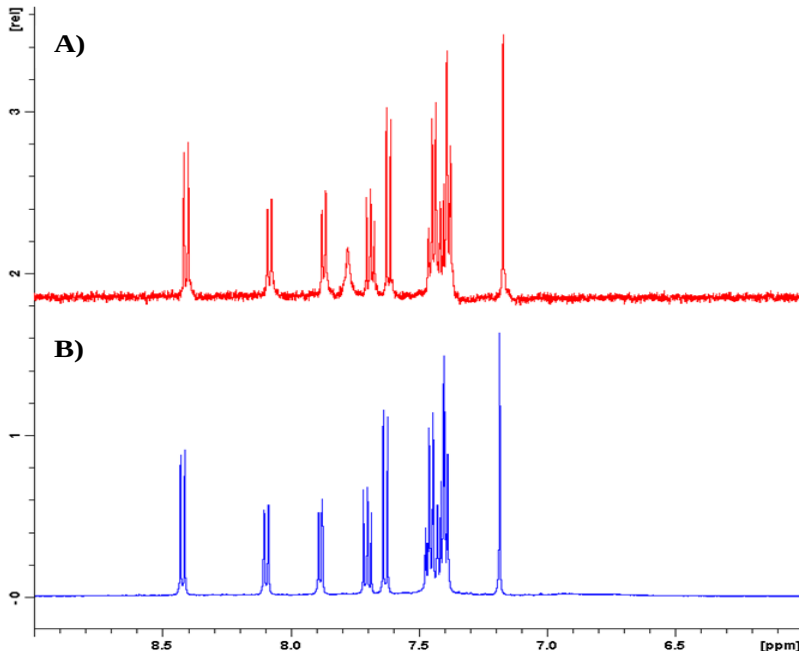


Figure 6.8: Identification of anigorufone (**1**) based on the comparison of ^{1}H NMR spectrum of authentic compounds.
A) The ^{1}H NMR spectrum of authentic compound anigorufone (**1**).
B) ^{1}H NMR spectra of the pHPLC peak corresponding to the aHPLC peak at the R_t 51.65 min.

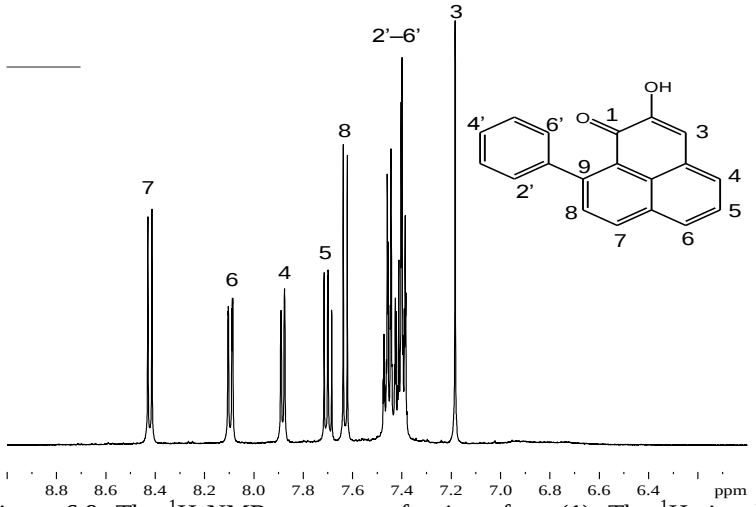


Figure 6.9: The ^{1}H NMR spectrum of anigorufone (**1**). The ^{1}H signals are numbered corresponding to their positions in the structure of the anigorufone (**1**).

The spectra of reference compounds were available in the collection at Max-Planck-Institute for Chemical Ecology. The ^{1}H NMR spectra of anigorufone (**1**) is presented in the Figure 6.9.

The pHPLC peak corresponding to the aHPLC peak eluted at 51.7 min was unambiguously identified as anigorufone (**1**) based on its clear ^{1}H NMR spectrum (Fig. 6.9). Anigorufone (**1**) is the main and the most abundant phenylphenalenone-type secondary metabolite found in Yangambi km5. This is also the case in Grande Naine. The high purity and the quantity of the anigorufone (**1**) enabled the measurement of the quantity of the compound using sensitive balance. The quantity of anigorufone (**1**) extracted from the characteristic small lesions covering only smaller areas of Yangambi km5 roots was 7.8 mg/kg of roots. The quantity of anigorufone (**1**) extracted from the large lesions covering most area of the Grande Naine roots was only 3.9 mg/kg of roots.

The pHPLC peak corresponding to the aHPLC peak eluted from 36 to 38 min was found to be comprised of three phenylphenalenones namely 4-phenyl-1*H*,3*H*-benzo[*de*]isochromene-1,3-dione (**8**), irenolone (**5**) and hydroxyanigorufone (**2**) based on the analysis of the ^{1}H NMR spectrum. By integrating the signals of the doublets belong to H4 of compound **8** and H7 of **5** and **2**, the relative ratio for the amount of the three metabolites present in the necrotic lesions were assessed. In Yangambi km5, the relative ratio of **8**, **5** and **2** was determined as 1.7:1.6:1.0 but in Grande Naine, the ratio was 1.0:1.3:3.5.

Yangambi km5 showed a slightly different phytochemical profile with more secondary metabolites than Grande Naine. Based on the ^{1}H NMR analysis, two 9-phenylphenalenone-types: anigorufone (**1**) and hydroxyanigorufone (**2**), the 4-phenylphenalenone-types: irenolone (**5**) and 4-phenyl-1*H*,3*H*-benzo[*de*]isochromene-1,3-dione (**8**) were detected in both Yangambi km5 and Grande Naine.

The 4-phenylphenalenone and the isomeric form of the anigorufone (**1**), isoanigorufone (**4**) was detected in Yangambi km5 but not in Grande Naine. Similarly two other phenylphenalenones, (2*S*,3*R*)-2,3-dihydro-2,3-dihydroxy-9-phenylphenalen-1-one (**3**) and anigorootin (**9**) were detected in Yangambi km5 and not detected in Grande Naine. The methylirenolone (**6**) detected in Grande Naine was not detected in Yangambi km5.

The UPLC-MS analysis also showed the molecular weight matching the nine phenylphenalenones which are presented in Table 6.2. Additionally, the UPLC-MS analysis allowed the detection of traces of 2-methoxy-1*H*-phenalen-1-one (**10**) in Yangambi km5.

6.1.3.6. LDI-MSI

LDI-MSI made it possible to detect the localised presence of the phenylphenalenones in the necrotic regions. The LDI mass spectra showed prominent signals for the presence of the UV-absorbing-secondary metabolites in the necrotic lesions of Grande Naine (Fig. 6.10A) and Yangambi km5 (Fig. 6.10C). The regions of necrotic lesions were strikingly different from the healthy, uninfected surrounding regions for the presence of secondary metabolites. The LDI mass spectra of uninfected regions showed no prominent signal for the presence of the phenylphenalenones (Figs 6.10B & D).

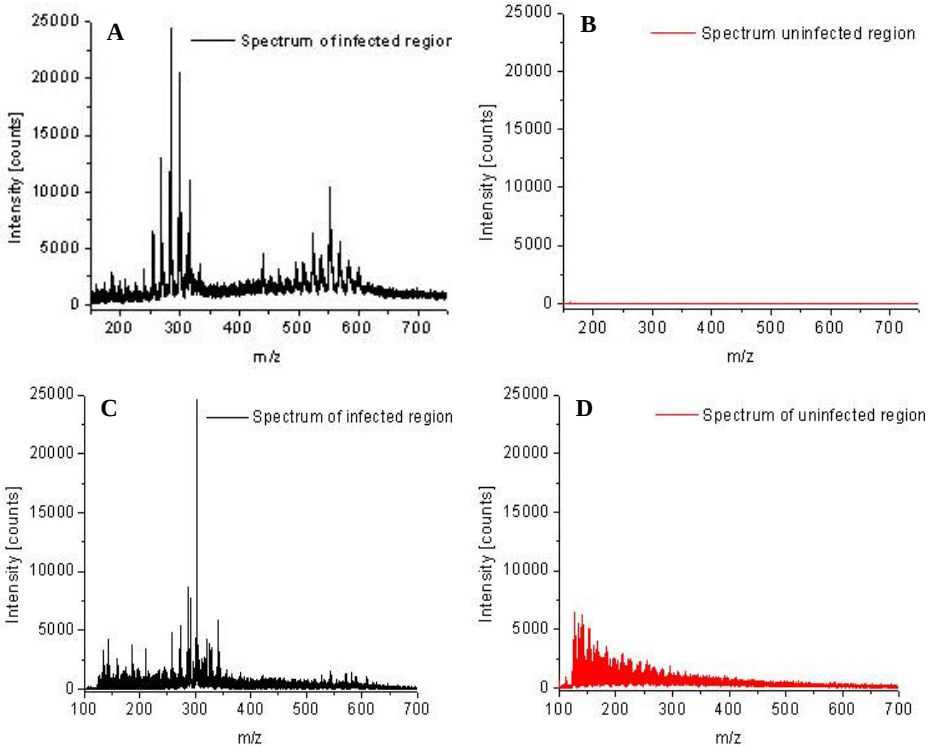


Figure 6.10: The LDI mass spectra of *Musa* roots. A) The *Radopholus similis*-infected region of Grande Naine. B) Uninfected region of Grande Naine. C) The *R. similis*-infected region of Yangambi km5. D) Uninfected region of Yangambi km5.

The LDI mass spectrum of the uninfected regions of Yangambi km5 showed high background effects (Fig. 6.10D). The heat profile for the *m/z* value of 271 as an arbitrarily chosen example verified the high intensity of the *m/z* value of 271 in the region of the necrotic lesions in opposite to the surrounding healthy tissue regions (Fig. 6.12). The *m/z* values 271, 273, 287, 289, 301 were detected in Yangambi km5 (Fig. 6.11) and in Grande Naine (Fig. 6.13).

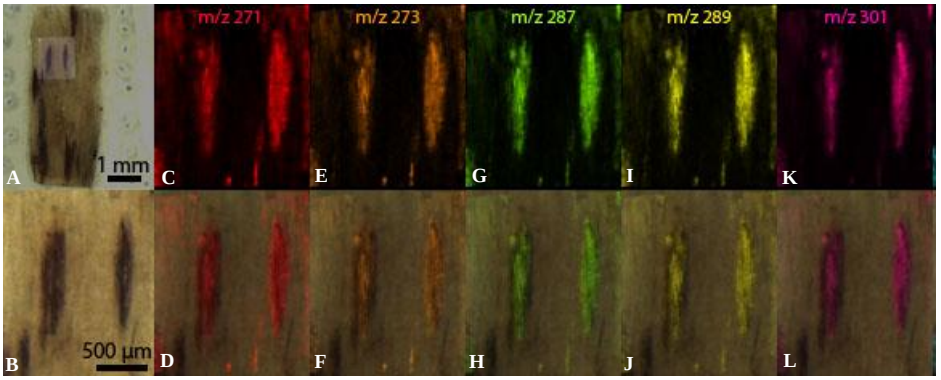


Figure 6.11: Mass images of the necrotic lesions on Yangambi km5 caused by *Radopholus similis* infection.
A) Optical image of the lesions on Yangambi km5 roots. B) The region of the lesions subjected for LDI-MSI. The molecular image of section B for the *m/z* C) 271 E) 273 G) 287 I) 289 and K) 301. The overlay of optical image B and the corresponding molecular images for the *m/z* values D) 271 F) 273 H) 287 J) 289 and L) 301.

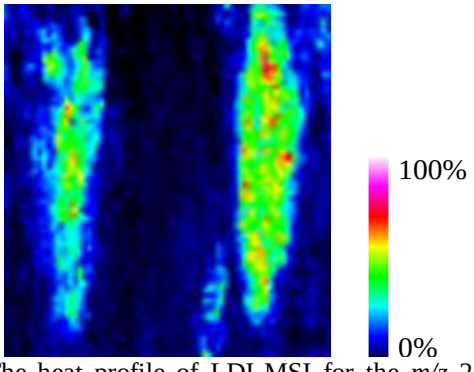


Figure 6.12: The heat profile of LDI-MSI for the *m/z* 271 in the necrotic lesions of Yangambi km5 roots showing the distribution of anigorufone only in the necrotic lesion. The blue coloured region represents absence of anigorufone (**1**).

Signals for compound **1** and **4** (*m/z* 271.08, $[M-H]^-$), compound **8** (*m/z* 273.06, $[M-H]^-$), compounds **2** and **5** (*m/z* 287.08, $[M-H]^-$), compounds **3** and **7** (*m/z* 289.09, $[M-H]^-$) were detectable in necrotic root lesions of Yangambi km5 (Fig. 6.11). Similarly, signals for compound **1** (*m/z* 271.08, $[M-H]^-$), compound **8** (*m/z* 273.06, $[M-H]^-$), compounds **2** and **5** (*m/z* 287.08, $[M-H]^-$), and compound **3** (*m/z* 289.09, $[M-H]^-$) were detected in necrotic root lesions of Grande Naine (Fig. 6.13). As described before, these compounds were identified using a series of the following analytical analyses of the necrotic root extracts: HPLC, ^{1}H NMR and UPLC-MS analysis.

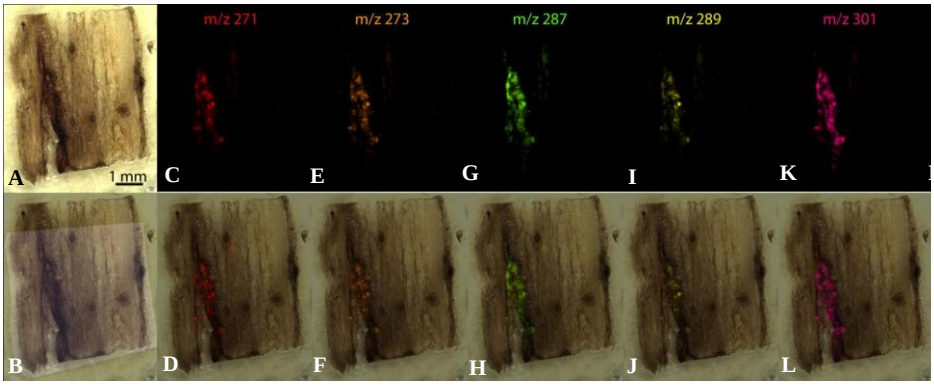


Figure 6.13: Mass images of the necrotic lesions on Grande Naine caused by *Radopholus similis* infection.
A) Optical image of the lesions on Grande Naine roots B) The region of the lesions subjected for LDI-MSI. The molecular image of section B for the *m/z* C) 271 E) 273 G) 287 I) 289 and K) 301. The overlay of optical image B and the corresponding molecular images for the *m/z* values D) 271 F) 273 H) 287 J) 289 and L) 301.

6.1.4. DISCUSSION

Our study provides clear evidences for the induction of phenylphenalenone-type secondary metabolites in response to *R. similis* infection in *Musa* spp. Moreover, phenylphenalenones are confirmed as phytoalexins due to their nematode infection-induced biosynthesis in the plants and their strong anti-nematode properties (see Chapter 6.2). In our study, we developed a novel strategy for a multidimensional approach combining analytical techniques such as HPLC, ^{1}H NMR, UPLC-MS and LDI-MSI for the structural identification of the secondary metabolites and to understand the cellular distribution in the plant system. This strategy is also applicable to study other plant-pathogen interactions. The aHPLC analysis of separate root extracts from lesions and healthy plants and the LDI-MS imaging illustrated the highly localised presence of phenylphenalenones in the nematode-infected lesions. No signals were detected for the presence of phenylphenalenones in the healthy regions of the infected roots or in the uninfected plant roots. The LDI-MSI technique was successful in imaging the UV absorbing *i.e.* condensed aromatic ring containing secondary metabolites at high spatial resolution in *A. thaliana* and *Hypericum* sp. (Hölscher *et al.*, 2009). For the first time, phenylphenalenones were successfully imaged in nematode-infected *Musa* roots.

Anigorufone (**1**) was identified as the most abundant phenylphenalenone-type secondary metabolite present in the lesions of Yangambi km5. Furthermore, it is well localised in the necrotic lesions. Interestingly, anigorufone (**1**) was also the most active compound against *R. similis* in the *in vitro* bio-assays causing the highest percentage nematode motility inhibition (see Chapter 6.2). Nematode mortality was observed at high concentrations (see Chapter 6.2). The anti-nematode property was positively correlated with the concentration of the phenylphenalenones (see Chapter 6.2). It is evident that the anigorufone (**1**) plays a key role in inhibiting *R. similis* reproduction in Yangambi km5. Anigorufone (**1**) was previously reported as a constitutive compound in *Anigozanthos* spp. (Cooke & Thomas 1975; Hölscher & Schneider, 1997) and as phytoalexin in the *R. similis*-resistant *Musa* cultivar Pisang Jari Buaya (Binks *et al*., 1997). Infection with fungal pathogens has shown to induce anigorufone (**1**) synthesis in *Musa* spp. (Kamo *et al*., 1998, 2000; Otálvaro *et al.*, 2007; Jitsaeng, 2009).

Anigorufone (**1**) was also found as a main phenylphenalenone in Grande Naine in our study. However, the quantity of anigorufone (**1**) in Grande Naine was only about half the quantity in Yangambi km5. Observation of the lesions demonstrated the highly concentrated accumulation of the secondary metabolites in small lesions of Yangambi km5; Accumulation of phytoalexins in larger necrotic areas resulting in lower concentration in Grande Naine. Hence, apart from higher quantity, the concentration per unit area is far higher in Yangambi km5 compared to Grande Naine. The high quantity and high concentration could create a more toxic cellular environment to the nematodes in Yangambi km5.

The localised synthesis of the phytoalexins at high concentration in the nematode-infected cells demonstrates the high degree of regulation in delivering the nematode toxins to the target cells. This tight, highly regulated synthesis and localised accumulation of the toxic metabolite in a very limited area at high concentration in the nematode-infected cells can be the key mechanism by Yangambi km5 to limit *R. similis* reproduction. The high concentrations of anigorufone (**1**), isoanigorufone (**4**) and hydroxyanigorufone (**2**) were shown to inhibit the nematode motility (see Chapter 6.2). The reduced motility and mortality probably limits the spread of the nematode to only a small area. Future studies could aim at finding the mode of action of anigorufone (**1**) and other active phenylphenalenones on nematode motility inhibition and toxicity.

Secondary metabolites are often found to accumulate in specific plant tissues at high concentrations (Opitz *et al*., 2003; Hölscher & Schneider, 2007; Wuyts *et al*., 2007; Bednarek & Osbourn, 2009). This accumulation in such compartments should be regulated in a highly sophisticated manner, because some secondary metabolites are phytotoxic if they are mislocalised (Yazaki *et al*., 2008). This tightly regulated expression only at the nematode-infected tissues or feeding sites shows their role in the plant's defense against nematodes.

Disease resistance is largely determined by the rapidity and magnitude of the synthesis or accumulation of the defence products instead of their presence or absence in the plants (Kuć, 1995; Wuyts *et al*., 2007). The speed of the phytoalexins accumulation and specific localisation at the infection sites of resistant plants result in concentrations far in excess of inhibitory to the invading pathogens (Mert-Türk, 2002). Phytoalexins were also shown to be specifically deployed to the sites of pathogenic attacks by vesicle-mediated trafficking (Field *et al*., 2006; Boller & He, 2009). The efficient cargo of phytoalexins is progressively studied as a key factor in making the plant resistant to diseases (Field *et al*., 2006; Cheung & De Vries, 2008; Kwon *et al*., 2008). It was reported that the most spectacular manifestation of plant resistance is the synthesis of phytoalexins in a limited area at the site of infection (Tronchet *et al*., 2010).

Phenylphenalenones were reported as inducible compounds of *Musa* spp. by pathogenic fungal infections such as *M. fijiensis* on leaves (Luis *et al*., 1993; 1995; Otálvaro *et al*., 2007), *F. oxysporum* in roots and rhizomes (Luis *et al*., 1994; 1996; 1997), *C. musae* on fruits (Kamo *et al*., 1998, 2001; Otálvaro *et al*., 2007) and by the nematode *R. similis* in roots (Binks *et al*., 1997). However, few reports showed that phenylphenalenones such as 4'-dehydroxyirenolone (Luis *et al*., 1999) and hydroxyanigorufone (**2**) (Kamo *et al*., 2000) were present in the healthy tissues of *Musa* spp. at very low concentrations. They possibly play a role as phytoanticipins. Moreover these phytoanticipin concentrations were shown to increase after infection with pathogens (Kamo *et al*., 2000).

The other phenylphenalenones isolated in our study were previously reported from *Musa*. Irenolone (**5**) and hydroxyanigorufone (**2**) were isolated as phytoalexins from the leaves of Grande Naine infected with *M. fijiensis* (Luis *et al*., 1993) and in rhizomes infected with *F. oxysporum* (Luis *et al*., 1996). 4-(4-hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromene-1,3-dione (**7**) was reported in unripe fruits infected by *C. musae* (Hirai *et al*., 1994). (*2S,3R*)-2,3-dihydro-2,3-dihydroxy-9-phenylphenalen-1-one (**3**), isoanigorufone (**4**), methylirenolone (**6**)

and anigorootin (**9**) were reported in *Musa* spp. as natural compounds or in response to fungal infections (Luis *et al*., 1995; Kamo *et al*., 1998; Otálvaro *et al*., 2007; Jitsaeng *et al*., 2010).

Methoxyanigorufone was reported as the major compound in Yangambi km5 rhizomes (Otálvaro *et al*., 2007). The two perinaphthenone type (phenalenone) compounds, 2-hydroxy-1*H*-phenalenone and 2-methoxy-1*H*-phenalenone (**10**) were previously reported from Yangambi km5 rhizomes (Otálvaro *et al*., 2007). With the exception of traces of 2-methoxy-1*H*-phenalenone (**10**) detectable by UPLC-MS technique, the other main compounds were not isolated from the nematode-infected roots in our study. The growing conditions and nematode infection in our study might be the reason for the different phytochemical profile of the Yangambi km5 roots.

Phenylphenalenones show a high potential in protecting monocot plants from biotic stresses. However they are relatively less explored. Treating *in vitro* banana roots with non-pathogenic yeast cultures of *Sporobolomyces salmonicolor* has been shown to induce phenylphenalenone-type compounds in the roots (Jitsaeng & Schneider, 2010). Chemical elicitors such as kanamycin and jasmonic acid also induced the synthesis of phenylphenalenones in *Musa* (Luis *et al*., 1993, Jitsaeng, 2009). The underlying reason for the enhanced tolerance to diseases due to endophytic colonization may also be attributed to enhanced phenylphenalenones biosynthesis. Future studies on the possibility of enhancing the cellular concentration and improved localisation of anigorufone (**1**) and other phenylphenalenones in Grande Naine roots will be highly fascinating to see if these changes may make Grande Naine also resistant to *R. similis* reproduction. Although metabolic engineering of secondary metabolites pathway for enhancing the disease resistance could be very challenging, a feasible strategy for is previously presented and discussed (Dixon, 2001).

6.1.5. CONCLUSION

Our study provides clear evidence for the induction of phenylphenalenone-type secondary metabolites in *Musa* spp. in response to *R. similis* infection. Nine phenylphenalenone-type phytoalexins were structurally identified from the necrotic tissues of the banana roots. The phenylphenalenones showed a highly localised presence in the nematode-infected necrotic regions of the banana roots. The phenylphenalenones were not detected in the healthy regions. Anigorufone (**1**) was identified as the most abundant phenylphenalenone-type secondary metabolite present in the necrotic lesions. It is evident that anigorufone (**1**) plays a key role in limiting *R. similis* reproduction in the

resistant *Musa* cultivar Yangambi km5. Anigorufone (**1**) was also a major compound in the susceptible *Musa* cultivar Grande Naine. However, the concentration per unit area was very high in Yangambi km5 compared to Grande Naine. This may have created a more toxic cellular environment to the nematode. Phenylphenalenones show a high potential in protecting monocot plants from biotic stresses.

CHAPTER 6.2: ANTI-NEMATODE PROPERTIES OF THE PHENYLPHENALENONES

6.2.1. INTRODUCTION

Studies on identifying phytochemicals with anti-nematode properties have been gaining interest among nematologists (Akthar & Mahmood, 1994; Chitwood, 2002; Wuyts *et al*., 2006). Phytochemical-based nematode management strategies could provide economically viable and environmentally safer alternatives to the conventional nematicides or they could serve as models for developing new nematicides (Chitwood, 2002).

Phytoalexins elicited in nematode-resistant plants are generally anticipated to have anti-nematode properties. However, knowledge on the mode of action, the biologically active concentrations and the effective exposure time are vital to determine the efficiency and feasibility of practical applications of phytochemicals. Phenylphenalenones biosynthesis is elicited in *Musa* plants in response to biotic stresses (Luis *et al*., 1993; Kamo *et al*., 1998). Biosynthesis of phenylphenalenones has become evident as a response to nematode infection in resistant as well as susceptible *Musa* cultivars (see Chapter 6.1). Hence it is our primary interest to discover the role of these nematode infection-site-specific compounds in the plant's defense against nematodes.

The antimicrobial properties of phenalenones were reported previously (Winters *et al*., 1977; Qin *et al*., 2006). Antifungal properties of phenylphenalenones against the major fungal pathogens of *Musa* spp. such as *Fusarium oxysporum* f. sp. *cubense*, *Colletotrichum musae* and *Mycosphaerella fijiensis* were also reported (Luis *et al*., 1996, 1998; Kamo *et al*., 1998; Quiñones *et al*., 2000; Lazzaro *et al*., 2004; Otálvaro *et al*., 2007). But to our knowledge, there is no study performed so far on the anti-nematode properties of the phenylphenalenones.

The objective of this part of our study was to identify the anti-nematode properties of the phenylphenalenone-type phytoalexins using *in vitro* bio-assays on the motility of *R. similis* as well as on the toxicity effect of the compounds on *R. similis*.

6.2.2. MATERIALS AND METHODS

6.2.2.1. Experimental set-up

Two series of *in vitro* bio-assay experiments were carried out. The first series of experiments were conducted with 13 different phenylphenalenones, each at two concentrations (50 and 100 ppm) to test their effects on *R. similis* motility. In the second experiments, the dosage effect of anigorufone (**1**) on *R. similis* motility was tested using a gradient of six concentrations (10, 20, 40, 50, 100 and 150 ppm). All experiments were repeated twice to confirm the results.

6.2.2.2. Chemicals

All phenylphenalenones used in the bio-assays were extracted and purified using pHPLC from *Musa* spp. and *Anigozanthos* spp. at the Max-Planck-Institute for Chemical Ecology. Compounds were dissolved in aqueous ethanol (1%). Stock solutions of 200 µg/ml (ppm) were prepared aseptically and stored in small portions at -20 °C.

6.2.2.3. Nematodes

A population of *R. similis* originally isolated from banana roots in Uganda was used in the experiments. This population from Uganda was characterized by a high reproductive fitness (Fallas *et al*., 1995). This population was maintained and multiplied monoxenically on sterile alfalfa callus tissues at 25±1 °C in the dark (Elsen *et al*., 2001). To obtain inoculum, nematodes were collected from the callus tissues or from the medium by rinsing with sterile distilled water. Only 6-weeks-old nematode cultures were used to prepare the inoculum of adults and juveniles of *R. similis*.

6.2.2.4. Effect of phenylphenalenones on *R. similis* motility bio-assay

Thirteen different phenylphenalenones were tested for their anti-nematode properties using *in vitro* bio-assays. The molecular structures of the compounds tested in the bio-assays are provided in the annexes. Nematode motility was assayed in two concentrations (50 and 100 ppm). Each treatment was replicated three times and for each compound, the assay was repeated at least twice. The experiment was performed in 24-well-plates under aseptic conditions. The negative control consisted of the compound solvent, 1% ethanol. A sterile distilled water control was also included to test the solvent effects on motility.

At the onset of experiment, an aliquot of 100 µl sterile nematode suspension containing 80-100 adults and juveniles of *R. similis* was added to

each well prior to the addition of the compounds. The exact numbers of motile and immotile nematodes was counted in each well using an inverted compound microscope (40×). The stock solution (200 ppm) of the compounds and sterile distilled water were added into each well according to their concentration. Plates were incubated in the dark at 25±1 °C. The numbers of quiescent (immotile) nematodes were counted after 24, 48 and 72 h of incubation.

The percentages of quiescent nematodes were calculated relative to the number of actively moving and immotile nematodes at the onset of the experiment. Compounds were considered motility inhibitive when significantly ($P \leq 0.05$) more nematodes have become quiescent than in the solvent control at any assayed concentration.

6.2.2.5. Dosage effect of anigorufone (1) on *R. similis* motility bio-assay

The motility bio-assay was performed with a gradient of six different concentrations of anigorufone (**1**): 10, 20, 40, 50, 100 and 150 ppm. Each treatment was replicated six times and the bio-assay was repeated twice. The experimental methods were the same as the motility assay (6.2.2.4). Dose-dependent effects allowed the calculation of the IC_{50} values at 24, 48 and 72 h of incubation.

The IC_{50} value is defined as the concentration of a compound required to cause 50% of the total nematodes to become quiescent. Toxicity of the anigorufone (**1**) was tested when a minimum of 10% or more nematodes become quiescent in a treatment than in the solvent control. Nematicidal effects were checked by transferring the quiescent nematodes to sterile distilled water after 72 h incubation in anigorufone (**1**) followed by counting the number of live and dead nematodes under a fluorescence microscope at 96 h (Forge & MacGuidwin, 1989) combined with poking the nematodes.

6.2.2.6. Statistical data analysis

Statistical analysis was performed using STATISTICA version 9 (StatSoft, Tulsa, OK, USA). Data on motility assay were analyzed using Kruskal-Wallis analysis of variance by ranks. When the Kruskal-Wallis analysis of variance by ranks was significant, each group mean was compared with the negative control by multiple comparisons of mean ranks.

Data on dosage effect of anigorufone (**1**) on *R. similis* motility were analyzed by analysis of variance (ANOVA) provided the conditions of ANOVA (normal distribution and homogeneity of variance) were met. The

percentage quiescent nematodes were arcsin (x/100) transformed prior to the analysis. When significant differences ($P \leq 0.05$) were observed, Tukey's HSD test was applied for multiple comparisons of group means. The IC_{50} values were obtained by interpolating the corresponding percentage of quiescent nematodes *vs.* anigorufone (**1**) concentration in scatter plots.

6.2.3. RESULTS

6.2.3.1. Effect of phenylphenalenones on *R. similis* motility bio-assay

The effects of the 13 assayed phenylphenalenones on *R. similis* motility are listed in Table 6.3.

Table 6.3. Effect of the phenylphenalenone-type phytoalexins on the motility of *Radopholus similis*.

Compound	Effect on motility
Anigorufone (**1**)	I
Anigorootin (**9**)	I
(2*R*,3*R*)-2,3-Dihydro-2,3-dihydroxy-9-phenylphenalenone	I
Dihydroxyanigorootin	-
Hydroxyanigorufone (**2**)	I
4-Hydroxy-2-methoxy-9-phenylphenalenone	I
4-(4-Hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromene-1,3-dione (**7**)	I
Irenolone (**5**)	I
Isoanigorufone (**4**)	I
Methoxyanigorufone	I
Methylirenolone (**6**)	-
Monohydroxyanigorootin	I
Perinaphthenone	I

A compound was considered motility inhibitive (I) when it caused significantly ($P \leq 0.05$) high percentage of nematode quiescence compared to the negative control. Significance was tested according to Kruskal-Wallis analysis.
I: Inhibitory effect; -: No effect.

Eleven out of 13 compounds inhibited the nematode motility in at least one concentration at any of the observed time points. Only dihydroxyanigorootin and methylirenolone (**6**) did not show any significant inhibition on nematode motility at any assayed concentrations.

The extended results of the nematode motility bio-assay are presented in Table 6.4. Specific to mention are anigorufone (**1**), 4-hydroxy-2-methoxy-9-phenylphenalenone and isoanigorufone (**4**) that caused about 75% of *R. similis* to become quiescent. Anigorufone (**1**), hydroxyanigorufone (**2**) and monohydroxyanigorootin were causing nematode quiescence in all tested

concentrations starting from 24 h of incubation and the effect was either consistent or increased over time.

The anti-nematode activity displayed by anigorufone (**1**) was the strongest causing 89% of *R. similis* to become quiescent at 72 h of incubation in the compound. 4-Hydroxy-2-methoxy-9-phenylphenalenone, 4-(4-hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromene-1,3-dione (**7**) and isoanigorufone (**4**) also caused persistent and strong nematode quiescence at 100 ppm concentration. However, at a lower concentration (50 ppm), a significantly ($P \leq 0.05$) high percentage of nematode quiescence was observed only starting from 48 h of incubation compared to the negative control.

Anigorootin (**9**), (2*R*,3*R*)-2,3-dihydro-2,3-dihydroxy-9-phenylphenalenone, irenolone (**5**), methoxyanigorufone and perinaphthenone showed moderate anti-nematode activity as they caused a non-persistent nematode quiescence at only one of the tested concentrations. The solvent 1% ethanol exhibited no significant effect on nematode movement compared to the sterile distilled water.

A notable observation was made while counting the quiescent nematodes. The nematodes ingested anigorufone (**1**) in such large amounts that the ingested anigorufone (**1**) molecules were clearly visible inside the nematodes under light microscopic observation (Figs 6.14 & 6.15). This is due to the natural colour of anigorufone (**1**) and the natural transparency of nematode cuticle. No coloured spots were observed in the nematodes in the controls (Fig. 6.16).

Due to this interesting observation, the bio-assay with anigorufone (**1**) was repeated many times. The percentages of nematodes ingesting visible amounts of anigorufone (**1**) were variable from one experiment to the other. However, all the nematodes that ingested visibly large amounts of anigorufone (**1**) molecules were always dead (Fig. 6.15).

Table 6.4. Percentage quiescent *Radopholus similis* caused by the phenylphenalenones at two concentrations (conc.) after 24, 48 and 72 h incubation (n=3).

Compound	Conc. (ppm)	Quiescent nematodes (%)		
		24 h	48 h	72 h
Anigorufone (**1**)	100	70.0	86.1	89.1
	50	51.4	70.7	67.9
4-Hydroxy-2-methoxy-9-phenylphenalenone	100	38.1	76.7	75.9
	50	-	74.9	72.2
Isoanigorufone (**4**)	100	25.5	74.4	72.8
	50	-	71.9	68.8
Hydroxyanigorufone (**2**)	100	47.7	67.6	68.7
	50	22.3	55.4	69.3
4-(4-Hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromene-1,3-dione (**7**)	100	26.6	66.1	66.3
	50	-	54.7	65.2
Monohydroxyanigorootin	100	35.9	59.4	51.2
	50	23.7	57.8	54.3
Irenolone (**5**)	100	-	55.4	-
Anigorootin (**9**)	50	-	51.3	-
Methoxyanigorufone	100	41.5	-	-
Perinaphthenone	100	33.4	-	-
(2*R*,3*R*)-2,3-Dihydro-2,3-dihydroxy-9-phenylphenalenone	100	-	31.9	26.1

All the presented mean percentage quiescent nematodes are significantly ($P \leq 0.05$) different from the percentage quiescent nematodes present in the negative control according to Kruskal-Wallis analysis.

- : No significant inhibitory effect was observed.

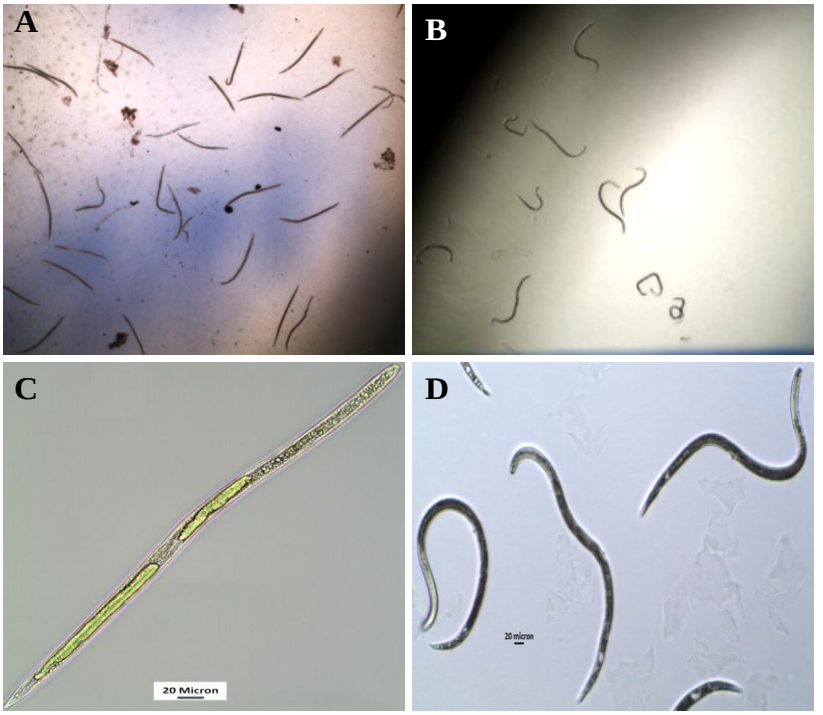


Figure 6.14: Light microscopic images of *Radopholus similis* during the bio-assay with anigorufone (**1**). A) Quiescent nematodes resulted from 24 h incubation in anigorufone (**1**). B) & D) Live, active nematodes present in the negative control, 1% ethanol after 24 h of incubation. C) Dead nematodes after ingesting anigorufone (**1**) molecules during the bio-assay; ingested molecules (yellow coloured in the gut) stored along the entire length of the nematode gut. Scale bar: C & D: 20 µm; A & B: 40 x magnification.

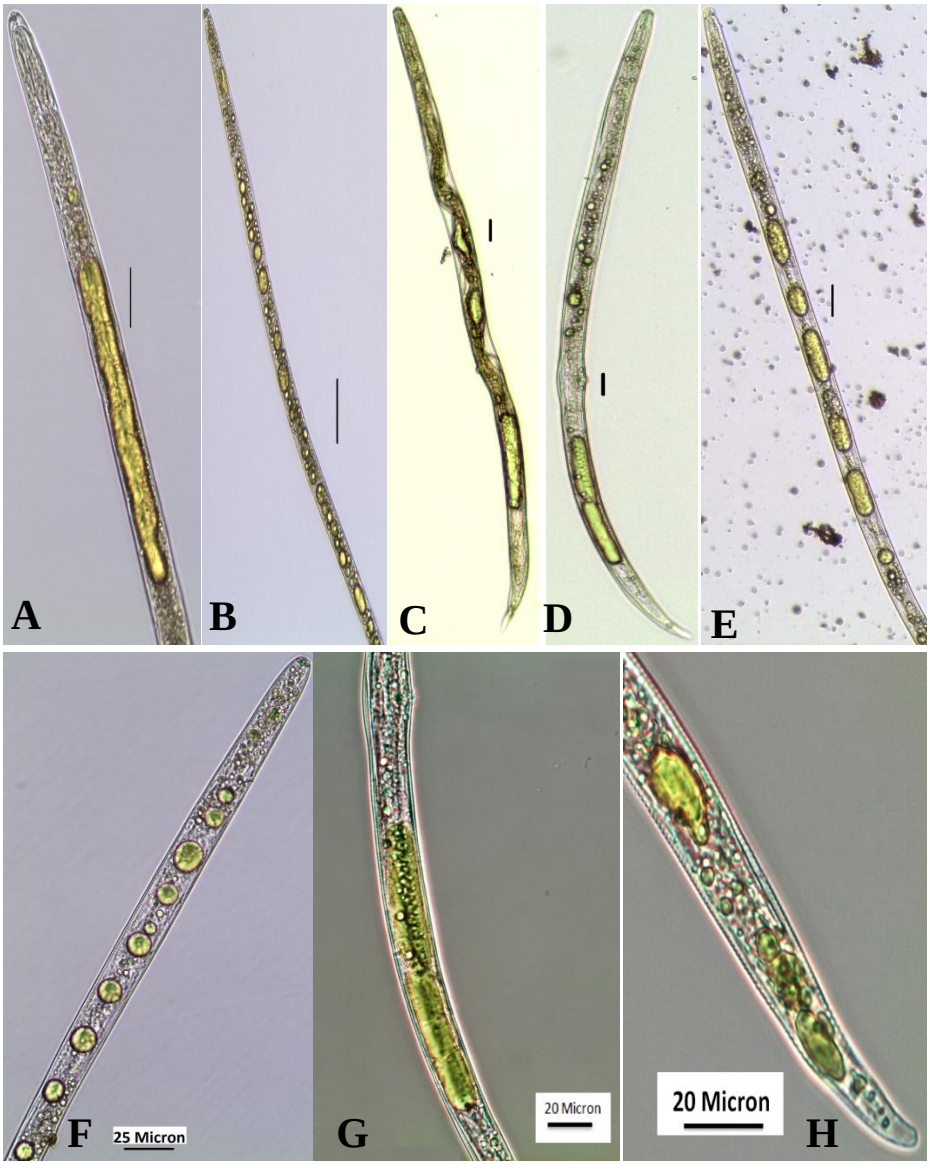


Figure 6.15: Light microscopic images of all life stages of *Radopholus similis* that had died because of ingesting anigorufone (**1**) during the bio-assays. A) Intense accumulation of the compound in a moulting juvenile. B) & F) Accumulation of anigorufone (**1**) in small vacuoles throughout the body of a juvenile. C) & D) Adult females; C) shows that the compound is present in the digestive tract not in the pseudocoelum. E) Male nematode. G) Female nematodes showing anigorufone (**1**) accumulated near the ovary. H) Anigorufone (**1**) accumulation at the end of the digestive tract but not in the tail. Scale bar: A: 25µm; B: 40µm; C, D & E): 20µm.

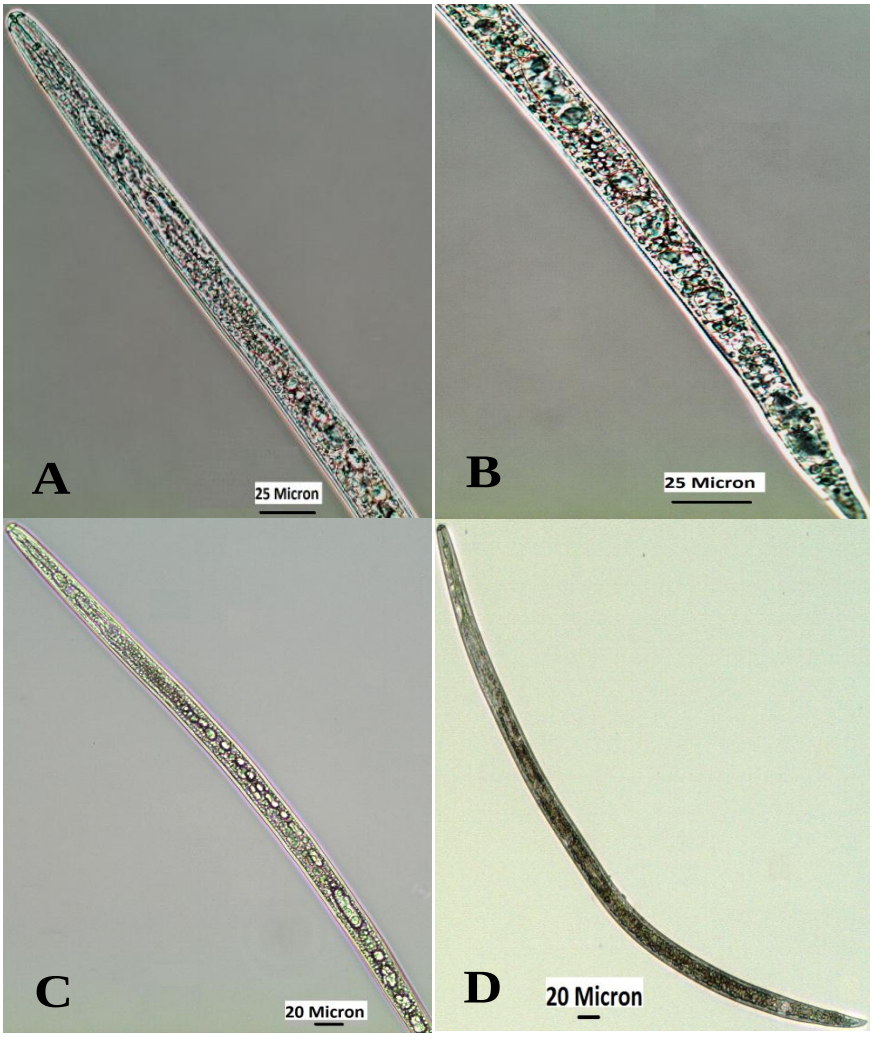


Figure 6.16: Light microscopic images of *Radopholus similis* from the negative control, 1% ethanol. Nematodes were temporarily fixed for imaging purpose. A) The head & B) the tail of a *R. similis* showing normal digestive tract. C) Juvenile & D) adult female *R. similis* after 72 h incubation in the negative control.

6.2.3.2. Dosage effect of anigorufone (1) on *R. similis* motility bio-assay

The dosage effects of anigorufone (**1**) on *R. similis* motility after 24, 48 and 72 h of incubation are listed in Table 6.5 and shown in the Figure 6.18. A sharp increase in the effect of concentration on nematode motility was observed after an initial lag phase (Fig. 6.17). At the lowest concentration of 10 ppm,

anigorufone (**1**) caused no effect on nematode motility after 24 to 72 h of exposure compared to the negative control, 1% ethanol.

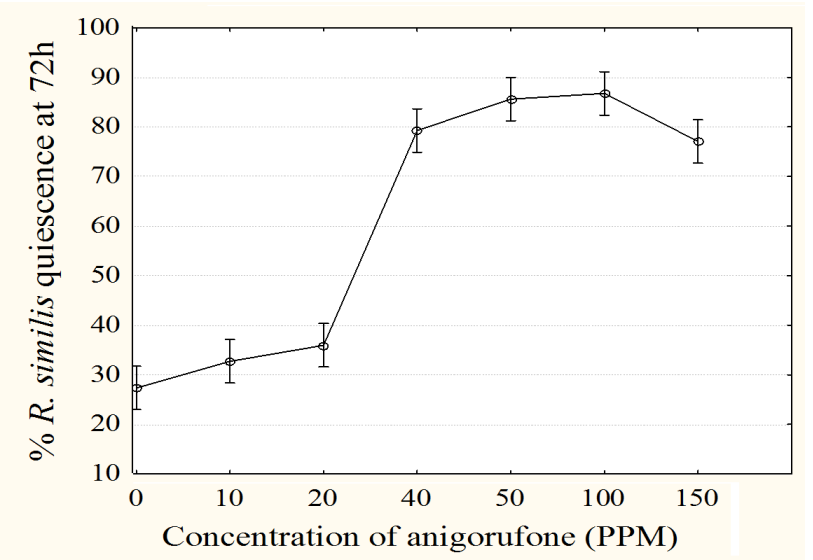


Figure 6.17: Percentage quiescent *Radopholus similis* caused by a gradient of six different concentrations of anigorufone (**1**) after 72 h exposure. Data are the averages of six replications. Error bars represent the confidence interval.

A slightly higher concentration of 20 ppm caused significant ($P \leq 0.05$) nematode quiescence after 24 h exposure compared to the negative control. But the effect was lost after 48 and 72 h. Significant ($P \leq 0.05$) and persistent percentages of quiescent nematodes were observed starting from 40 ppm concentration onwards compared to the negative control (Fig. 6.18 & Table 6.5).

At all the observed incubation times, anigorufone (**1**) showed the highest percentage of quiescence to *R. similis* at 100 ppm. Even a higher concentration of 150 ppm showed a slightly lower inhibition especially at 72 h where, the percentage quiescent nematodes was significantly ($P \leq 0.05$) lower than in 100 ppm (Fig. 6.18 & Table 6.5).

The inhibitory concentrations (IC) of anigorufone (**1**) which caused 50% of nematode quiescence (IC_{50}) values are listed in Table 6.6. Anigorufone (**1**) showed the strongest motility inhibition to *R. similis* at 72 h of incubation causing quiescence to 50% of the nematodes at a low concentration of 23 µg/ml (ppm).

Table 6.5. Percentage quiescent *Radopholus similis* caused by anigorufone (**1**) at six different concentrations over three days (n=6).

Concentration of anigorufone (ppm)	Quiescent nematodes (%)		
	24h	48h	72h
10	25.9 a†	30.2 a†	32.7 a†
20	31.4 a	28.6 a†	35.9 a†
40	50.2 b	68.6 b	79.3 bc
50	63.8 c	79.3 c	85.6 c
100	74.3 d	85.0 c	86.8 c
150	70.1 cd	82.8 c	77.0 b
1% ethanol	17.6	21.0	27.3

Means within each column followed by the same letters are not significantly ($P \leq 0.05$) different from each other according to Tukey's HSD test. Means followed by † are not significantly different from the mean percentage quiescent nematodes present in the negative control (1% ethanol).

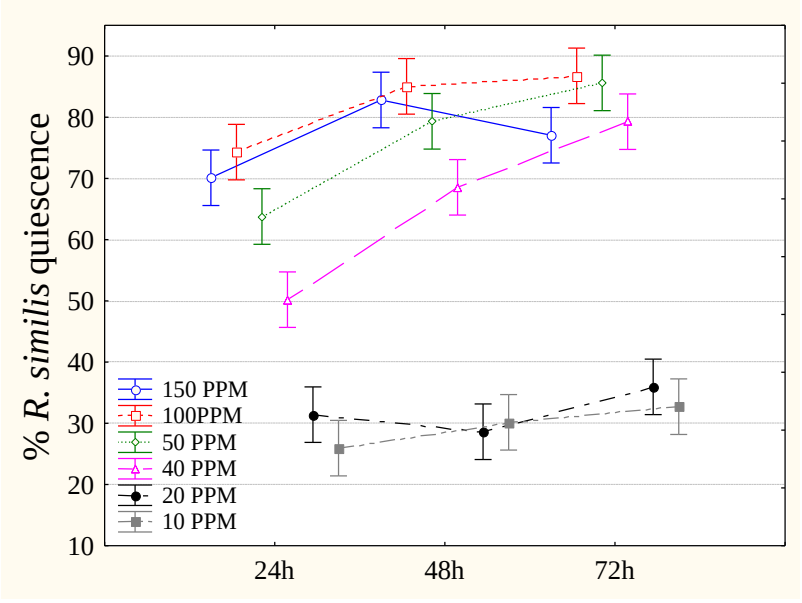


Figure 6.18: Percentage quiescent *Radopholus similis* observed during the motility bio-assay on a concentration gradient of anigorufone after 24, 48 and 72 h of incubation. Error bars represent the confidence interval (n=6).

Table 6.6. The motility inhibitive concentrations (IC_{50}) of anigorufone (**1**) on *Radopholus similis* after 24, 48 and 72 h of incubation.

Hours of incubation in anigorufone (**1**)	IC_{50} (µg/ml)
24	59
48	38
72	23

The IC_{50} values were obtained by interpolating the corresponding percentage of quiescent nematodes *vs.* anigorufone (**1**) concentration in scatter plots.

6.2.4. DISCUSSION

Biological activities of phenylphenalenones against bacteria, fungi, algae and diatoms have been previously reported (Winters *et al*., 1977; Kamo *et al*., 1998; Quiñones *et al*., 2000; Lazzaro *et al*., 2004; Qin *et al*., 2006; Otálvaro *et al*. 2007). To the best of our knowledge, our study presents the first evidence of anti-nematode properties of phenylphenalenones based on systematic bio-assays. Eleven of the 13 tested phenylphenalenones were active against the burrowing nematode *R. similis*. Especially anigorufone (**1**) along with 4-hydroxy-2-methoxy-9-phenylphenalenone and isoanigorufone (**4**) were remarkably powerful anti-nematode compounds assayed in our study. The nematode quiescence caused by anigorufone (**1**) was the strongest of all tested compounds.

Concentration of the compounds plays a key role in causing the quiescence as we could see that the increase in concentration of anigorufone (**1**) caused a sharp increase in the nematode quiescence after an initial lag phase. However, it also reached a stationary phase at higher concentrations. Starting from 50 ppm concentration onwards, it caused nematode mortality due to the ingestion of large amounts of anigorufone (**1**) molecules by the nematodes. The cellular concentration of the anigorufone in resistant plants could be much higher than the concentrations tested in the bio-assay. So the toxicity effect is very much applicable to the real time situation.

The intake of the anigorufone (**1**) molecules seems to be both oral and transcuticular as the anigorufone (**1**) molecules are found inside the moulting juveniles. It was observed that a few nematodes with high reserve food particles seem to avoid ingestion of the anigorufone (**1**) molecules as they sustain from the reserve food particles. Consequently, they stay alive at the early hours of exposure and/or at low concentrations. However, after a prolonged exposure such as after 48 or 72 h, they also tend to ingest the compound even at a lower concentration of 50 ppm.

At initial observation, the yellow coloured spots were distributed in small putative lipid-like globules. However, with time, the globules joined with each other and formed a channel of yellow globules accupying a large space of the nematode body volume. This might also imposs a large constraint in nematode body affecting the nematode viability. The possibility of anigorufone forming a complex with the nematode's putative lipid molecules could be of interest for future studies. Lipids are reported in the free-living nematode *Caenorhabditis elegans* (Klapper *et al*., 2011). The reason why the nematodes did not excrete but accumulate the anigorufone remains to be studied further. Biogenic amines and trace amines such as tyramine and octopamine were shown to affect the behaviour of free living nematode *Caenorhabditis elegans*. These amines were shown to inhibit the aversive response of *C. elegans* to 100% octanol (Wragg *et al*., 2007). Possibility of such behavioural modulation by the nematode-ingested phytoalexins influencing the behaviour of *R. similis* could be of interest to study in future. Lipophilic compounds are highly membrane disruptive (Cowan, 1999). Thus, there is also a possibility that the ingestion of the lipophilic anigorufone (**1**) by nematodes could be associated with the toxicity.

Bio-assays conducted by Quiñones *et al*. (2000) and Otálvaro *et al*. (2007) showed that anigorufone (**1**) was also a notable antifungal compound which significantly inhibited the mycelial growth but, moderately inhibited the spore germination of *M. fijiensis*, the causal agent of Black Sigatoka leaf disease in *Musa* spp. Anigorufone (**1**) was also active against *F. oxysporum* in dark but inactive in light (Lazzaro *et al*., 2004).

In previous reports, isoanigorufone (**4**) showed strong inhibition of *F. oxysporum* in the dark but was less active in light (Lazzaro *et al*., 2004). However, the same compound showed only mild and non-persistent inhibition of *M. fijiensis* (Quiñones *et al*., 2000).

Hydroxyanigorufone (**2**), 4-(4-hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromene-1,3-dione (**7**), and monohydroxyanigorootin were the next notably active anti-nematode compounds in our study. Antifungal properties of hydroxyanigorufone (**2**) were reported against key pathogens of *Musa* spp. A moderate to low inhibition of *M. fijiensis* (Quiñones *et al*., 2000; Otálvaro *et al*., 2007), a significant inhibition of *F. oxysporum* in light as well as in dark (Lazzaro *et al*., 2004) and a moderate inhibition of *C. musae* (Kamo *et al*., 1998) were caused by the hydroxyanigorufone (**2**). The photoactive toxicity of phenylphenalenones was shown to be caused by the production of singlet oxygen (Lazzaro *et al*., 2004).

Based on the results of a study on the anti-fungal activity of 14 different phenylphenalenones and their derivatives, Kamo *et al*. (1998) concluded that a phenolic-hydroxyl group is essential for this anti-fungal activity. Our study is not in agreement with this conclusion because the highly active anti-nematode compounds such as anigorufone (**1**), isoanigorufone (**4**) possess a phenolic group without any hydroxyl group. Further studies are required with structurally diverse compounds to understand the relationship between the structural similarities and anti-nematode property. The same study by Kamo *et al*. (1998) also suggested that the 4-phenylphenaelnone types could be more biologically active than the 9-phenylphenalenones types based on their observation that irenolone (**5**) inhibited the spore germination of *C. musae* more than hydroxyanigorufone (**2**). In contrast, in our study, the 9-phenylphenalenone, anigorufone (**1**) was more active against *R. similis* than the 4-phenylphenalenone, isoanigorufone (**4**). Similarly, hydroxyanigorufone (**2**) was more active than irenolone (**5**).

Irenolone (**5**), anigorootin (**9**) and methoxyanigorufone caused moderate but non-persistent quiescence of *R. similis*. Irenolone (**5**) was also less active against *M. fijiensis* and *C. musae* (Quiñones *et al*., 2000; Kamo *et al*., 1998). Methoxyanigorufone was highly active against *F. oxysporum* in the presence of light but inactive in the dark and moderately active against *M. fijiensis* (Otálvaro *et al*., 2007). Perinaphthenone was reported as a powerful anti-fungal compound: which inhibited the *M. fijiensis* significantly more than the fungicide benomyl. As perinaphthenone is an efficient singlet oxygen sensitizer, it was suggested that the high anti-fungal activity could be a result of the production of singlet oxygen (Quiñones *et al*., 2000). In contrast, in our study, perinaphthenone displayed very low activity against *R. similis* and it remained effective only until 24 h incubation.

The suggested mechanisms conferring the antibiotic properties of phenylphenalenones are DNA intercalation, radical formation and oxygen activation (Winters *et al*., 1977; Kamo *et al*., 1998; Quiñones *et al*., 2000). Lazzaro *et al*. (2004) provided evidence for photoactive toxicity of phenylphenalenones due to the production of singlet oxygen. However, light-induced toxicity could not function against nematodes due to the fact that nematodes live in dark and the phenylphenalenones are elicited in roots under dark conditions. The phenylphenalenones such as anigorufone (**1**), isoanigorufone (**4**) and hydroxyanigorufone (**2**) which displayed anti-fungal activity under the dark experimental conditions (Lazzaro *et al*., 2004) were the most active anti-nematode compounds in our study and the reverse (*i.e.* those displayed high anti-fungal activity in light are less active or inactive against

nematodes) is also true. Further investigations are needed to explain the mechanism and conditions required for the anti-nematode properties of the phenylphenalenones.

Laboratory *in vitro* bio-assays may have a limited relevance to the field or real time situations (Spence *et al.*, 2008). In real time plant-nematode interactions, enzymatic oxidation and polymerisation of individual compounds could occur resulting in increased or reduced toxicity. Nevertheless, the laboratory bio-assays are a simplified and practical version of the field conditions (Spence *et al.*, 2008). In our study, we focused mainly on the individual compound effects not on the whole extract and insufficient amount of compounds obtained from the plants limited us from performing extended toxicity, reversibility and dosage effect studies of all the interesting compounds. The individual compounds in the whole root extract could interact with each other synergistically or antagonistically (Chitwood, 2002). Elaborated future bio-assays using different combinations of the identified compounds will enhance the understanding on the synergism or antagonisms of the compounds.

Several practical applications could result from the identification of the anti-nematode phytochemicals. The compounds could be developed as plant-based nematicides or they could serve as models for developing nematicides based on the relationship between the chemical structure and the biological activity. However, extreme caution must be exercised in this application as certainly not all phytochemicals are safe for the environment, non-target organisms or humans (Chitwood, 2002). The most appealing way forward is to exploit the plant-nematode interactions to enhance the cellular synthesis and localisation of the most active anti-nematode compounds in the target cells of the susceptible plant roots.

6.2.5. CONCLUSIONS

From this study, we offered first evidence for the anti-nematode properties of phenylphenalenones. Anigorufone (**1**) was the most powerful anti-nematode compound assayed. At higher concentrations such as 50, 100 and 150 ppm of anigorufone (**1**), nematodes ingested large amounts of the compound causing mortality of the nematodes. 4-Hydroxy-2-methoxy-9-phenylphenalenone and isoanigorufone (**4**) are remarkably powerful anti-nematode compounds assayed in our study. Further studies could aid in developing these highly active anti-nematode phytochemicals for use in practical applications for nematode management.

CHAPTER 7

GENERAL CONCLUSIONS AND PERSPECTIVES

In our study, we evaluated and characterized the mechanism(s) of resistance to *Radopholus similis* (Cobb, 1893) Thorne, 1949, a migratory endo-parasitic nematode, in a selection of *Musa* genotypes. For this purpose, seven recently identified *R. similis*-resistant *Musa* genotypes were verified for their *R. similis* resistance under greenhouse conditions (Chapter 2). The well-known *R. similis*-resistant cultivars Yangambi km5 and Pisang Jari Buaya, and the *R. similis*-susceptible cultivar Grande Naine were included as references for comparison. The host response of these *Musa* genotypes to *Meloidogyne incognita* infection was also evaluated to examine if the *R. similis*-resistant genotypes are also resistant to the root-knot nematode *M. incognita* (Chapter 2). Resistance of Long Tavoy, Saba, Pisang Mas and Pora Pora to *R. similis* was confirmed. The *Musa* genotypes Long Tavoy, Saba and Yangambi km5 were shortlisted for characterization of the *R. similis* resistance. The *Musa* genotypes Vudu Papau and Pisang Mas were identified as resistant to *M. incognita*. Our study identified Pisang Mas as a *Musa* genotype with combined resistance to both *R. similis and M. incognita*. This is the first time that a *Musa* genotype has been found that is resistant to both these two major banana root pathogens.

The first major specific objective of our study was to find out at which phase of the nematode-plant interactions (*i.e.* pre- or post-infection) the resistance to *R. similis* was active. To achieve this objective, an autotrophic *in vitro* model system was developed to facilitate the study of host location and penetration behaviour of *R. similis* on the selected *R. similis*-resistant and susceptible *Musa* genotypes (Chapter 3). In autotrophic *in vitro* systems, plant shoots are exposed to open air allowing the plant to perform active photosynthesis through atmospheric carbon exchange. At the same time, the root system and the nematodes are maintained under strict *in vitro* conditions in a Petri dish. Due to the horizontal root growth in the Petri dishes, this system enables direct observation of the roots and the nematodes using a stereoscopic microscope. The autotrophic *in vitro* model system proved to be a very good model system and advantageous compared to the strict *in vitro* systems to study nematode attraction and penetration. Our direct microscopic observation of nematode attraction and migration showed no differences in host location of the *R. similis*-resistant genotypes Saba and Yangambi Km5, and the susceptible *Musa* genotype Grande Naine by *R. similis* females. Our novel two-compartment autotrophic *in vitro* set-up proved that the *R. similis* females showed no preference to migrate towards the roots of either the resistant (Yangambi km5) or susceptible (Grande Naine) *Musa* genotypes when a choice was given to migrate towards both plant roots at the same time. Also no differences were observed in penetration of the resistant and susceptible *Musa*

genotypes by *R. similis* females under autotrophic *in vitro* conditions. In conclusion, our studies using the autotrophic *in vitro* systems showed that no preformed resistance factors inhibit the nematode attraction, migration towards and penetration of the resistant *Musa* roots compared to the susceptible genotype Grande Naine.

Greenhouse experiments were conducted on the penetration, development and reproduction of *R. similis* on resistant and susceptible *Musa* genotypes. Our objective of this study was to understand whether the *R. similis* resistance operates pre-infectionally towards the nematode attraction and penetration or post-infectionally towards nematode development and reproduction (Chapter 4). No significant differences were observed in the number of *R. similis* penetrating the resistant *Musa* genotypes Long Tavoy, Saba and Yangambi km5, and the susceptible genotype Grande Naine in our study. But, the post-infectional nematode development and reproduction were highly impaired in the resistant *Musa* genotypes. The number of eggs, juveniles, males, females and the final population density were significantly higher in Grande Naine than in the *R. similis*-resistant *Musa* genotypes. On the basis of these observations, it appears that the mechanism of resistance in the investigated *Musa* genotypes to *R. similis* is induced after nematode penetration and that the preformed host resistance factors do not function strongly against nematode attraction, migration towards the roots, and penetration of the roots.

The second major specific objective of our study was to identify the phytochemicals involved in the resistance to *R. similis* in *Musa* genotypes. A preliminary phytochemical profiling was performed to localise and quantify lignin and total phenols in the *R. similis*-resistant and susceptible *Musa* genotypes (Chapter 5). Our results showed that *R. similis* infection increased the root secondary wall lignification of the endodermis and vascular bundles of resistant and susceptible *Musa* genotypes. This increased lignification is a general defense response to protect the vascular bundle to reduce damage to the plant. Extensive lignification was not found associated with the cortex cells that are directly involved in the defense with nematodes. Hence, lignification does not seem to be associated with resistance to nematode development, reproduction and multiplication. Preformed phenolic cells were not found to be related with nematode resistance in the studied *Musa* genotypes. The nematode infection has dramatically increased the total phenol contents of resistant plants. Phenolic secondary metabolites are major constituents of the nematode infected necrotic cells.

In the final part of our study to characterize the nematode resistance in *Musa* genotypes, more detailed analytical studies were carried out to identify the secondary metabolites that are induced in nematode infection sites (Chapter 6.1). Phytochemical profiling of Yangambi km5 and Grande Naine root extracts was carried out using a combination of analytical techniques namely HPLC, ^{1}H NMR, UPLC-MS and LDI-MSI analysis. The results of this study provided clear evidence for the induction of phenylphenalenone-type secondary metabolites in *Musa* spp. in response to *R. similis* infection. Nine phenylphenalenone-type phytoalexins were isolated and structurally identified from *R. similis*-infected necrotic tissues of the banana roots. The phenylphenalenones showed a highly localised presence only in the nematode-infected necrotic regions of the banana roots. The phenylphenalenones were not detected in the healthy regions. Anigorufone (**1**) was identified as the most abundant phenylphenalenone-type secondary metabolite present in the necrotic lesions. It is evident that anigorufone (**1**) plays a key role in limiting *R. similis* reproduction in the resistant *Musa* cultivar Yangambi km5. Anigorufone (**1**) was also a major compound in the susceptible *Musa* cultivar Grande Naine. However, the concentration of anigorufone (**1**) per unit area was very high in Yangambi km5 compared to Grande Naine. This higher concentration of anigorufone localised in few cells in the *R. similis*-resistant cultivar can create a more toxic cellular environment to the nematode compared to Grande Naine. Phenylphenalenones show a high potential in protecting monocot plants from biotic stresses. Hence we conclude that the presence of the phenylphenalenone-type phytoalexins at high concentrations localised in the hypersensitive-like necrotic cells of Yangambi km5 is highly associated with the mechanism of *R. similis* resistance.

The anti-nematode properties of the identified phenylphenalenones were assessed by *in vitro* bio-assays on nematode motility inhibition and nematode mortality (Chapter 6.2). Our results offered the first evidence for the anti-nematode properties of phenylphenalenones. Eleven out of thirteen tested phenylphenalenones were inhibitive to *R. similis* motility. Anigorufone (**1**), 4-hydroxy-2-methoxy-9-phenylphenalenone and isoanigorufone (**4**) are remarkably powerful anti-nematode compounds tested in our study causing quiescence of more than 75% of the nematodes. Hydroxyanigorufone (**2**) and monohydroxyanigorootin were also highly inhibitive to *R. similis* motility starting from 24 h of incubation and the effect was either consistent or increased over time. Anigorufone (**1**) was the most powerful anti-nematode compound assayed. At higher concentrations such as 50, 100 and 150 ppm of anigorufone (**1**), nematodes ingested large amounts of the compound causing mortality of

the nematodes. The ingested anigorufone was found accumulated in the nematode guts of all life stages of nematodes. Elaborated future bio-assays using different combinations of the identified compounds will enhance the understanding on the synergism or antagonisms of the compounds.

The identification of these anti-nematode phytochemicals offers many practical applications for nematode management. Though there are the options to either develop the phytochemicals as plant-based nematicides or to use them as models for developing nematicides, extreme caution must be exercised in this application as certainly not all phytochemicals are safe for the environment, non-target organisms or humans (Chitwood, 2002). The most appealing way forward is to exploit the plant-nematode interactions to enhance the cellular synthesis and localisation of the most active anti-nematode compounds in the target cells of the susceptible plant roots.

Future studies could explore the possibilities of enhancing the cellular concentration and improved localisation of anigorufone (**1**) and other phenylphenalenones in the roots of *R. similis*-susceptible commercially successful banana cultivars such as Grande Naine. A feasible strategy for metabolic engineering of secondary metabolites pathways for enhancing disease resistance has been previously discussed by Dixon (2001).

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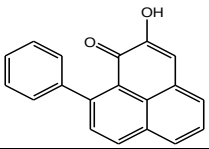
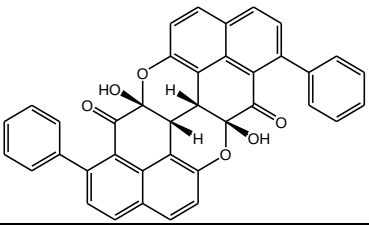
ANNEXES

Annex 1: **IUPAC name / trivial name and chemical structure of the phenylphenalenones tested in *in vitro* bio-assays.**

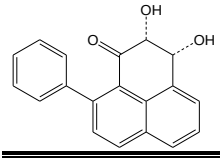
2-Hydroxy-9-phenyl-1*H*-phenalen-1-one / Anigorufone



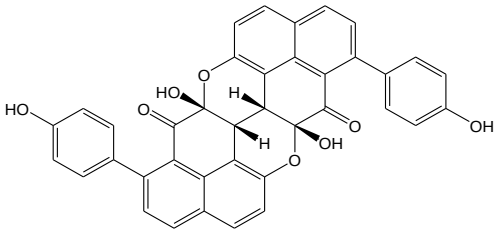
7b,14b-Dihydro-7a,14a-dihydroxy-6,13-diphenyl-(7*H*,14*H*)-diphenalen[2,3,3a,4-b,c,d:2,3,3a,4-g,h,i]pyrano[4,3-c]pyran-7,14-dione / Anigorootin



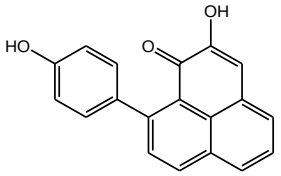
(2*R*,3*R*)-2,3-Dihydro-2,3-dihydroxy-9-phenylphenalen-1-one / Musanolone C



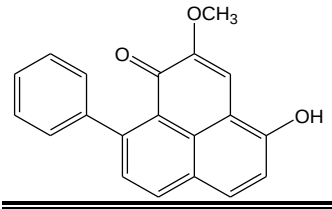
7b,14b-Dihydro-7a,14a-dihydroxy-6,13-di-(4-hydroxyphenyl)-(7*H*,14*H*)-diphenalen[2,3,3a,4-b,c,d:2,3,3a,4-g,h,i]pyrano[4,3-c]pyran-7,14-dione / Dihydroxyanigorootin



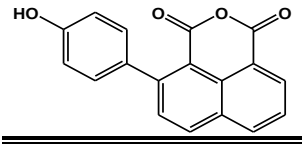
2-Hydroxy-9-(4-hydroxyphenyl)-1*H*-phenalen-1-one / Hydroxyanigorufone



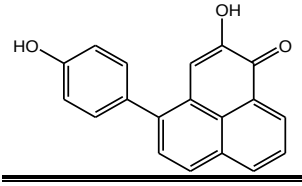
4-Hydroxy-2-methoxy-9- phenalen-1-one



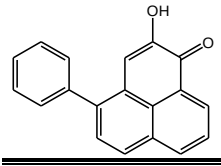
4-(4-Hydroxyphenyl)-1*H*,3*H*-benzo[*de*]isochromene-1,3-dione



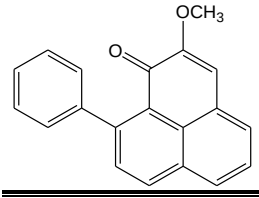
2-Hydroxy-4-(4-hydroxyphenyl)-1*H*-phenalen-1-one / Irenolone



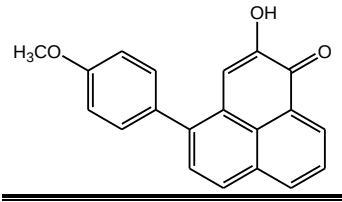
2-Hydroxy-4-phenyl-1*H*-phenalen-1-one / Isoanigorufone



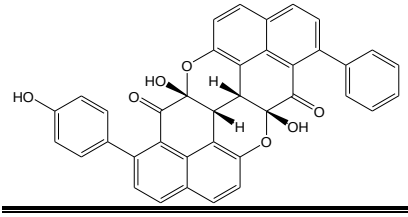
2-Methoxy-9-phenyl-1*H*-phenalen-1-one / Methoxyanigorufone



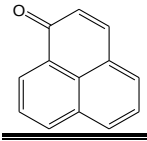
2-Hydroxy-4-(4-methoxyphenyl)-1*H*-phenalen-1-one / Methylirenolone



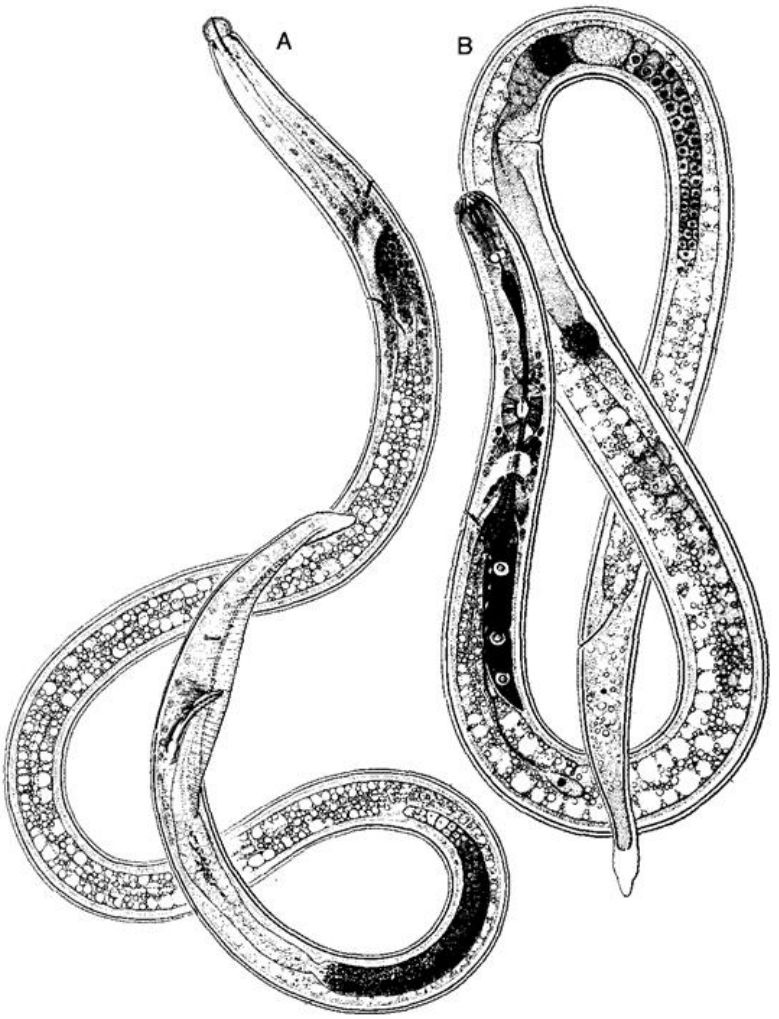
7b,14b - Dihydro - 7a,14a - dihydroxy-6 -(4 -hydroxyphenyl)-13-phenyl-(7*H*,14*H*)-diphenalen[2,3,3a,4-b,c,d:2,3,3a,4-g,h,i]pyrano[4,3-c]pyran-7,14-dione / Monohydroxyanigorootin



1*H*-Phenalen-1-one / Perinaphthenone



Annex 2: Diagram of *Radopholus similis* A) male and B) female nematodes. Source: Siddiqi, 2000.



Annex 3: CURRICULUM VITAE

Suganthagunthalam DHAKSHINAMOORTHY (SUGANTHA)
13241 Warrensville Cove, Alpharetta, GA 30004, USA.
Email: **Suganthamoorthy@gmail.com**

RESEARCH EXPERIENCE

Sep 2006 - Nov 2011 - **PhD research scholar** of Interfaculty Council for Development Cooperation (IRO), K.U. Leuven, Belgium

- Identified anti-nematode phytochemicals in banana by phytochemical profiling and *in vitro* toxicity assays
- Developed autotrophic *in vitro* model system to study plant-nematode interactions

Dec 2005 - Aug 2006 - **Senior research fellow** at Indian Institute of Horticultural Research (IIHR), Bangalore, India

- Identified media additives to scale up the production of fungal and bacterial bio-control microorganisms

Sep 2004 - Oct 2005 - **M.Sc. scholar** of Flemish Interuniversity Council (VLIR), University of Gent, Belgium

- Developed a split-root model system to study the systemic effects of arbuscular mycorrhizal fungi on nematodes

Aug 2003 - Aug 2004 - **Research fellow** at National Research Centre for Banana (NRCB), Tiruchirappalli, India

- Isolated, cultured, and mass multiplied the bio control microorganisms and nematodes and studied the effects of the microorganisms on nematode pathogenesis

EDUCATION

2011 - **PhD in Bioscience Engineering, Katholieke Universiteit Leuven, Belgium**
Thesis title: Characterization of *Radopholus similis* resistance in *Musa* spp. with emphasis on phytochemical analysis

2005 - **M.Sc. in Nematology, University of Gent, Belgium**
Thesis title: Induced bio-protective effect of arbuscular mycorrhizal fungi towards plant parasitic nematodes: a systemic or localized effect?

2003 - **B.Sc. in Agriculture, Tamil Nadu Agricultural University** (TNAU), Coimbatore, India

TRAINING

Apr 2009 - June 2009 - **Short term scientific mission** to Max-Planck-Institute for Chemical Ecology, Jena, Germany. Funded by European Cooperation in Science and Technology (COST)

- Performed secondary metabolites profiling using HPLC, NMR, MS and MS imaging

HONOURS

1. The greatest distinction for academic excellence, University of Gent (UGent), Gent, Belgium, 2005
2. Dr. M. Karunanidhi and R.S. Paroda award for the best B.Sc. (Ag) student in Trichy campus, Tamil Nadu Agricultural University, 2003
3. Dr. K. Rajukannu Medal for best outgoing student who got highest OGPA (Overall Grade Point Average) in B.Sc. (Ag) program during 2002-2003
4. Mr. Ibrahim Mohammed Doka Mohammed and Kenana sugar company Ltd., prize for best B.Sc. (Ag) student during the year 2002-2003

5. Meenashi ammal Medal for highest OGPA in agricultural botany courses from I to VI semester, 2002
6. Tmt. R. Santha medal for having obtained highest OGPA in all agronomy courses in B.Sc. (Ag) 2002-2003
7. AVR Sundaram trust Salem award for having obtained highest OGPA in horticultural courses in B.SC. (Ag) during the year 2002-2003
8. 1995-96 batch students endowment medal for best Student in agricultural economics in B.Sc. (Ag), during the year 2002-2003
9. Western electronic and scientific works prize award for highest OGPA I to VI semester in B.Sc. (Ag), 2002

LIST OF PUBLICATIONS

A. Articles

1. Suganthagunthalam, D., Elsen, A. and De Waele, D. 2010. Identification of combined resistance to *Radopholus similis* and *Meloidogyne incognita* in *Musa* germplasm. *International Journal of Nematology* 20, 19-26.
2. Suganthagunthalam, D., Galon, E. J., Elsen, A. and De Waele, D. 2009. Host plant resistance in *Musa* spp. to the burrowing nematode: Pre or Post-infectional? *Communications in Applied and Biological Sciences* 74: 39-43.
3. Suganthagunthalam, D., Galon, E. J., Elsen, A. and De Waele, D. 2009. Pathogenesis of *Radopholus similis* in an incompatible interaction with *Musa* spp. *Communications in Applied and Biological Sciences* 74: 541-544.
4. Suganthagunthalam, D., Swennen, R., De Waele, D. and Elsen, A. 2008. Host plant response of burrowing nematode resistant *Musa* genotypes to the root-knot nematode, *Meloidogyne incognita*. *Communications in Applied and Biological Sciences* 73: 219-222.

B. Abstracts/Proceedings

1. Knop, K., Hölscher, D., Suganthagunthalam, D., Elsen, A., Crecelius, A., Heckel, D., Schneider, B. and Schubert, U. 2009. LDI imaging of phenylphenalenones in infected banana tissues and pest. *Proceedings of 18th International Mass Spectrometry Conference*, Bremen, Germany, 30 August to 04 September 2009.

2. Suganthagunthalam, D., Elsen, A. Swennen, R., De Waele, D. and Hölscher, D. 2009. Phytoalexins involved in incompatible plant-nematode interactions. Third annual meeting of COST 872, "Nemagenics – Exploiting genomics to understand plant-nematode interactions". Toledo, Spain, 25-28 May 2009.
3. Suganthagunthalam, D., Galon, E. J., Elsen, A. and De Waele, D. 2009. Pathogenesis of *Radopholus similis* in an incompatible interaction with *Musa* spp. *61st International Symposium on Crop Protection*, Gent, Belgium, 19th May 2009.
4. Suganthagunthalam, D., Galon, E. J., Elsen, A. and De Waele, D. 2009. Host plant resistance in *Musa* spp. to the burrowing nematode: Pre or Post-infectional? *15th PhD Symposium on Applied Biological Sciences*, Leuven, Belgium, 6th November 2009.
5. Suganthagunthalam D., Elsen A. and De Waele D. 2008. Detailed evaluation of *in vitro*-propagated *Musa* spp. for resistance against the burrowing nematode, *Radopholus similis*. In: *Book of abstracts of 9th International Congress of Plant Pathology*, Torino, Italy, 24-29 August 2008. (Eds). Porta-Puglia A. & Gonthier P. *Journal of plant pathology* 90, S2.345.
6. Suganthagunthalam D., Swennen R., De Waele D. and Elsen A. 2008. Host plant response of burrowing nematode resistant *Musa* genotypes to the root-knot nematode, *Meloidogyne incognita*. *14th PhD symposium on applied biological sciences*, Gent, Belgium, 15th September 2008.
7. Suganthagunthalam D., Elsen A. and De Waele D. 2007. Characterization of resistance in *Musa* against the burrowing nematode, *Radopholus similis*. *First annual meeting of COST 872, "Nemagenics – Exploiting genomics to understand plant-nematode interactions*". La Colle-Sur-Loup, France, 9-11 May 2007.

GRADUATE / UNDERGRADUATE STUDENTS' THESIS SUPERVISION

1. *Els Heylen*. (Belgium) Studies on burrowing nematode resistant *Musa* genotypes to understand their resistant mechanisms. Bioscience and Engineering, Katholieke University of Leuven, Belgium 2009-10.
2. *Katemani Mdili*, (Tanzania) Characterization of transgenic banana shoots with RNAi constructs against *Radopholus similis*. M.Sc. Nematology, University of Gent, 2009-10.
3. *Erwin Jamilo Galon*. (Philippines) Occurrence of host plant resistance in *Musa* genotypes to the burrowing nematode: Pre or post-infectional? M.Sc. Nematology, University of Gent, Belgium 2008-09.
4. *Kahpui Mariama Salifu*. (Cameron) Biochemical and histochemical studies on the burrowing nematode resistant *Musa* genotypes – A step towards understanding the mechanisms of nematode resistance. M.Sc. Nematology, University of Gent, 2008-09.